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Responses of Unicellular Cyanobacteria to  
Changes in their Carbon Regime

by

Stephen A. Bloye

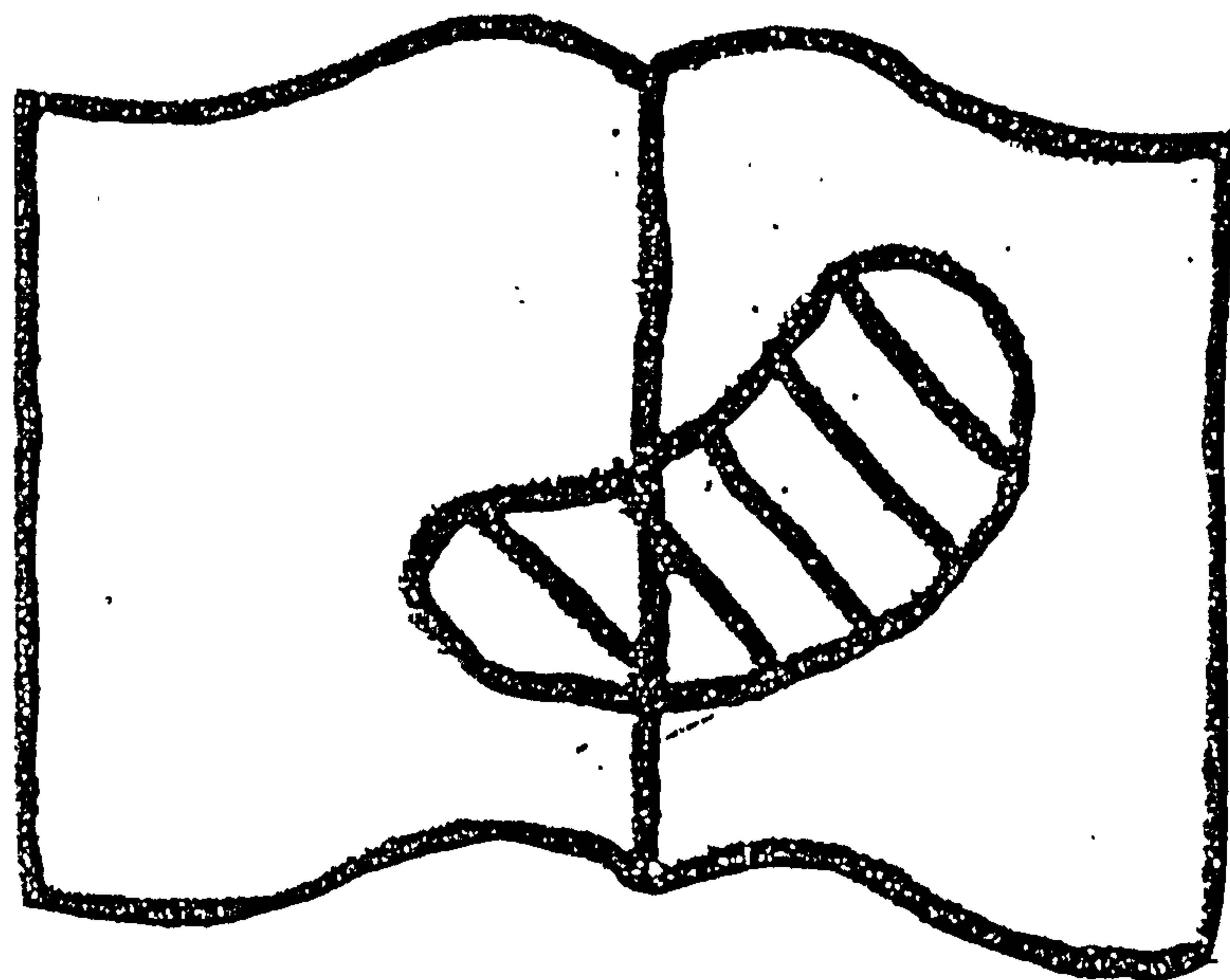
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Abbreviations

Ap <sup>r</sup>	- ampicillin resistant
ATP	- adenosine triphosphate
CA	- carbonic anhydrase
Cl	- inorganic carbon
Cm <sup>r</sup>	- chloramphenicol resistant
cpm	- counts per minute
DEO	- diepoxyoctane
DIC	- dissolved inorganic carbon
DOG	- 2-deoxy-D-glucose
DNase	- deoxyribonuclease
dpm	- disintegrations per minute
EDTA	- ethylenediaminetetra-acetic acid, disodium salt
EZ	- ethoxyzolamide
h	- hour
kb	- kilobase pairs
Km <sup>r</sup>	- kanamycin resistant
min	- minute
Mr	- relative molecular weight
MUG	- methyl umbelliferyl- -D-galactosidase
NTG	- N-methyl-N'-nitro-N-nitrosoguanidine
OD	- optical density, absorbance
OMG	- 3-O-methyl-D-glucopyranose
PAGE	- polyacrylamide gel electrophoresis
PMSF	- phenylmethylsulphonylfluoride
psi	- pounds per square inch
RNase	- ribonuclease



## XVIII

rpm	- revolutions per minute
RuBisCO	- D-ribulose 1,5 biphosphate carboxylase oxygenase
RUBP	- ribulose 1,5 biphosphate
SDS	- sodium dodecyl sulphate
TCA	- trichloroacetic acid
Tris	- Tris(hydroxymethyl)aminoethane
v/v	- concentration, volume by volume
w/v	- concentration, weight by volume

Declaration

The work contained in this thesis was the result of original research conducted by myself, with the exception of those instances where the contribution of others has been specifically acknowledged. All sources of information have been specifically acknowledged by way of reference.

None of the work contained in this thesis has been used in any previous application for a degree.

Stephen A. Bloye

"It is not without justice that I claim indulgence for this work, and I beg that no one will charge me with negligence, if he finds that I have passed over some illustration. For who could prove equal to the task of examining all the records which have come down to us....."

Frontius, Stratagems, I

## Summary

The rate of inorganic carbon (Ci) uptake and RuBisCO activity was examined in batch culture, in a range of unicellular cyanobacteria grown under different carbon regimes. There was no significant increase in RuBisCO activity seen in CO<sub>2</sub>-limited cultures. All of the species capable of growth in freshwater were found to possess, to varying degrees, an active, saturable, mechanism for the transport of Ci. Three oceanic, phycoerythrin containing unicellular cyanobacteria did not possess a Ci uptake mechanism following growth under high and low CO<sub>2</sub>. This may be related to the nature of the environment which these organisms inhabit.

Addition of sodium bicarbonate and glucose to low CO<sub>2</sub>-grown *Synechocystis* PCC6803 resulted in a rapid decay in Ci uptake, with a  $t_{0.5}$  for the decay of 1.15 h. Addition of the non-metabolizable glucose analogues 3-O-methyl-D-glucose (OMG) and 2-deoxy-D-glucose to low CO<sub>2</sub>-grown *Synechocystis* PCC6803 resulted in different effects on the cells ability to transport Ci. Addition of OMG resulted in no significant decline in the Ci uptake mechanism, whereas addition of DOG led to a similar decay in Ci uptake to that seen following addition of glucose. From these results it is suggested that the different structures of the analogues are responsible for the differences seen, and that build-up of a pool of glucose-6-phosphate is a signal for the inactivation of the Ci uptake mechanism in *Synechocystis* PCC6803.

Examination of the Ci uptake mechanism and RuBisCO activity in DIC and light-limited chemostats of *Synechococcus* PCC7942 resulted in the finding that cultures which were DIC-limited for growth were not DIC-limited with respect to the Ci uptake mechanism. RuBisCO activity increased in three distinct stages as the external DIC concentration fell. The 42 kD cytoplasmic membrane polypeptide was present in organisms isolated from DIC-limited chemostats with no appreciable Ci uptake mechanism.

The *in vivo* and *in vitro* phosphorylation pattern of *Synechocystis* PCC6803 was also examined in response to growth under different carbon regimes. Low CO<sub>2</sub>-grown cultures contained few phosphopolypeptides. Addition of sodium bicarbonate and glucose to low CO<sub>2</sub>-grown cells led to the appearance of a number of phosphopolypeptides over a similar time course seen to the decay of the Ci uptake mechanism. The glucose analogues DOG and OMG had no effect on the phosphorylation pattern in low CO<sub>2</sub>-grown cells. *In vitro* kinase experiments on cell-free extracts resulted in a number of polypeptides becoming phosphorylated, some corresponding on SDS-PAGE to those seen *in vivo*. The *in vitro* phosphorylation pattern could be regulated by altering the redox potential and addition of KUBP.

At the molecular level, the construction and use of the *lacZ* promoter probe pLACPB2 led to the identification of a number of presumptive CO<sub>2</sub>-regulated promoters.

## Chapter 1

### Introduction



### 1.1 Cyanobacteria: a general introduction

Formerly called blue-green algae because of their characteristic pigmentation, the cyanobacteria are one of the major groups of Gram negative prokaryotes and are the most diverse and ubiquitous group of photosynthetic prokaryotes.

They are found in practically all habitats which support life, from under the permanent ice cover of the antarctic to hot springs, where they are the only oxygen evolving photosynthetic organisms (Brock, 1969). They occur in static and running water, freshwater and marine habitats, where they form an important component of the oceanic phytoplankton crop (Waterbury et al., 1979; Morris and Glover, 1981). A number of aquatic-terrestrial species have also been recorded, and although the majority of these show a distinct preference for habitats of high relative humidity, soil cyanobacteria are also found in tropical and arid regions (Fogg et al., 1973).

Morphologically, cyanobacteria show a great diversity of form, unrivalled amongst the prokaryotes. Although the cell envelope of cyanobacteria exhibits the characteristics of Gram-negative bacteria, the peptidoglycan layer is relatively thick, and Jurgens and Weckesser (1985) have suggested a simple classification into the Gram-negative type might not be justified. Structurally they range from simple unicellular rods or cocci that reproduce by binary fission or budding, through to the more complex subgroups which have a filament of cells (trichome) as the structural unit, reproduction occurring through the production of hormogonia, short motile chains of cells.

Certain filamentous cyanobacteria are capable of other forms of cellular differentiation. When transferred to medium lacking or limited in fixed nitrogen, specialised cells called heterocysts are produced, which maintain an anaerobic environment that facilitates the fixation of atmospheric nitrogen by the enzyme nitrogenase. (see Wolk, 1980). As a strategy for nitrogen fixation, heterocyst formation is a feature unique to cyanobacteria, and the characteristic cellular spacing along a filament, together with the precise biochemical and genetic changes that occur during their development provide a model system for studying cellular differentiation (Adams and Carr, 1981).

Other important differentiated cell types produced by certain genera of cyanobacteria in response to environmental stimulation include hair cells, which develop with the onset of phosphate deficiency, and akinetes. Akinetes develop from vegetative cells, tend to be more resistant to dessication and cold than vegetative cells, and germinate to produce vegetative cells in response to improved environmental conditions. In this respect they are comparable to bacterial spores (see Nichols and Adams, 1982).

All cyanobacteria are aerobic photoautotrophs capable of a "plant-like" oxygenic photosynthesis. Phylogenetically, cyanobacteria more closely resemble the chloroplasts of eukaryotic algae, particularly rhodophytes and cryptomonads than the bacteria so far studied (Bonen and Doolittle, 1976). The photosynthetic apparatus is also similar structurally and

functionally to that found in the eukaryotic chloroplast, where with the exception of *Gloeobacter*, which lacks thylakoids (Rippka *et al.*, 1974), a series of flattened, membranous sacs or thylakoids develop in the protoplast, which are the site of the light harvesting pigments, the photochemical reaction centres and the photosynthetic electron transport chain. This similarity gave rise to the endosymbiotic theory for the origin of chloroplasts, first enunciated by Mereschowsky (1905), brought to the attention of modern biology by Margulis (1975) and aptly summarised by Doolittle (1982) " protoeukaryote first engulfed and later enslaved free living oxygenic photosynthetic prokaryotes of which modern plastids are the degenerate descendants ". Bryant (1986) gives a detailed review of the cyanobacterial and higher plant photosynthetic apparatus. The major light harvesting pigments are the phycobiliproteins (PBP's). These pigments are assembled into phycobilisomes, attached in regular rows to the outer surface of the thylakoids. The light energy they absorb is transferred primarily to photosystem II, so is responsible for photosynthetic oxygen production, but can also feed photosystem I (Jones and Myers, 1964). Two classes of PBP's are universal constituents of cyanobacteria, phycocyanins (PC) and allophycocyanins (AP) (Cohen-Bazire and Bryant, 1982) . Many cyanobacteria also synthesize a third class of PBP, the phycoerythrins (PE). Amongst the cyanobacteria that do contain phycoerythrins, some are capable of complementary chromatic adaptation, whereby the spectral quality of light received markedly affects the PE:PC



ratio (see Bogorad, 1975). This permits cyanobacteria to exploit light filtered by other overlaying pigmented organisms, in particular the green light unused by most algal groups (van Liere and Walsby, 1982). Light regulation of gene expression in cyanobacteria is also demonstrated by gas vacuole formation, where by regulation of gas vacuole content, buoyancy is controlled to allow planktonic cyanobacteria to select a position of optimum irradiance (Walsby, 1987).

Most cyanobacteria are obligately photoautotrophic, although a few are additionally capable of anoxygenic photosynthesis (Garlick et al., 1977). The ability of some species to perform oxygenic and facultative anoxygenic photosynthesis allows these organisms to occupy an interlinking position in the phototrophic world, and predominate in habitats alternating between photoaerobic and photoanaerobic conditions (Padan and Cohen, 1982). Some are also capable of growing photoheterotrophically (Rippka et al., 1979) or chemoheterotrophically (Ingram et al., 1973), however under all of these facultative conditions growth is usually much slower than when grown photoautotrophically, dark grown organisms retaining high levels of photosynthetic pigments (Hoare et al., 1971). *Nostoc* sp. MAC for instance, one of the most rapidly metabolizing chemoheterotrophic cyanobacteria, is poised for immediate photosynthetic metabolism when supplied with light (Bottomley and Van Baalen, 1978).

Genome complexity, transcription and translation are typical of that seen in other bacteria (see Herdman et al., 1979). The molecular biology of cyanobacteria has advanced enormously over

the past decade, using the techniques developed in other prokaryotes (for reviews see Shestakov and Reaston, 1987; Tandeau de Marsac and Houmard, 1987).

The processes of nitrogen fixation have long been studied by molecular biologists in the hope of developing new symbiotic associations or even transferring the genes for nitrogenase activity to higher plants. The photoautotrophic physiology of cyanobacteria and the fact they are contemporaries of the eukaryotic chloroplast, means they provide a model system for analysing gene expression in response to light and  $\text{CO}_2$  availability and can serve as a source of genes for chloroplast engineering (see Loffelhardt, 1987).

The biotechnology industry has recently turned it's attention to cyanobacteria (Craig and Reichelt, 1986). Certain cyanobacteria may be useful in the industrial production of ammonia (Kirby et al., 1986), whilst the mass culture of cyanobacteria as a food source for humans and cattle has re-emerged (Klausner, 1986).

D-ribulose 1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39

(RuBisCO), the key enzyme in  $\text{CO}_2$  fixation and thus plant productivity, has long been a target of molecular biologists, since the oxygenase activity of RuBisCO has long been regarded by plant physiologists as the most important restraint on plant productivity. Site-specific mutations have led to further insights into the catalytic mechanism of the enzyme (Gutteridge, 1986), and may eventually lead to the selective abolishment of the oxygenase activity. Cyanobacteria, as will be seen in the following sections, when grown under ambient  $\text{CO}_2$  conditions do

not photorespire, due to the possession of an inorganic carbon (Ci) concentrating mechanism. This mechanism has recieved a lot of attention, and once identified and characterised, transfer of the genes responsible for it's operation to commercially important higher plants will be of obvious biotechnological importance. The current state of cyanobacterial molecular genetics with regard to the Ci concentrating mechanism is discussed in section 6.1.2.

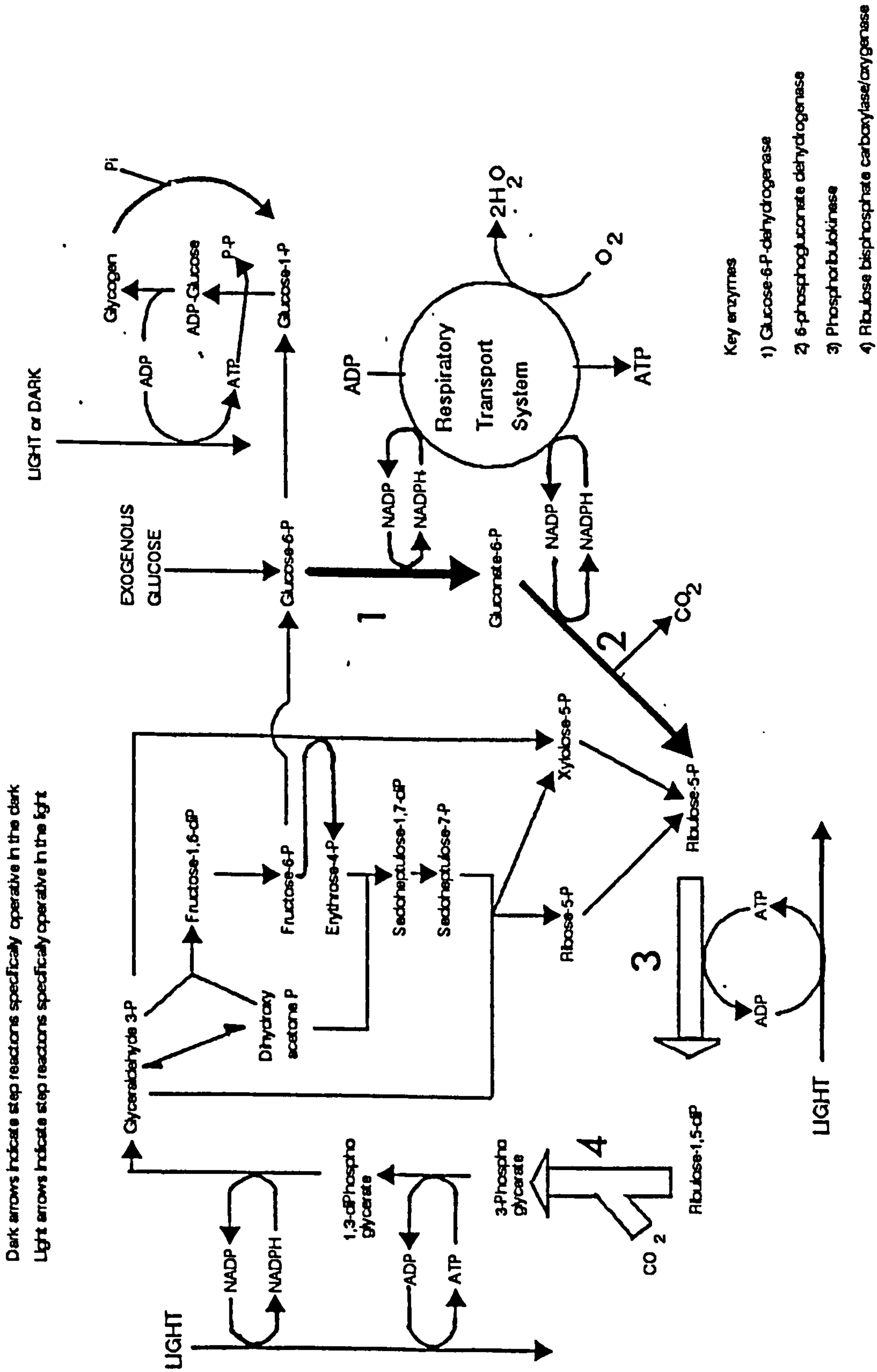
## 1.2 Modes of cyanobacterial carbon metabolism

### 1.2.1 Light and dark metabolism in cyanobacteria

As photoautotrophs, all cyanobacteria are capable of growth on carbon dioxide as the sole carbon source, using the chemical energy of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) derived from the absorbtion of light, to convert  $\text{CO}_2$  into reduced carbon compounds (Tabita, 1987). The primary route for the reduction of carbon dioxide into cell carbon in cyanobacteria is the reductive pentose phosphate or Calvin pathway (see Figure 1.1). There are two enzymes unique to this cycle, phoshoribulokinase and RuBisCO. RuBisCO catalyzes the actual fixation of carbon dioxide onto RUBP, whilst the action of phoshoribulokinase regenerates RUBP. This cycle can be visualised as producing one molecule of glucose for every 6 molecules of carbon dioxide fed into it, but the fixed carbon can, in actual fact be taken out in a variety of forms. The operation of the cycle represents a major energy commitment from the cell in the form of ATP and  $\text{NADPH}_2$ , which



Fig. 1.1 : Primary routes of carbon metabolism in cyanobacteria.



are generated as a consequence of the photochemical reactions. The evidence for the operation of this pathway in cyanobacteria has been established by a number of workers by using brief exposures of  $^{14}\text{CO}_2$  to organisms during growth, followed by kinetic analysis of the  $^{14}\text{C}$  flow into metabolic intermediates. (Kindel and Gibbs, 1963; Pelroy and Bassham, 1972).

The fixation of carbon dioxide into PGA may not be the only mechanism by which cyanobacteria assimilate carbon dioxide. Recently an assimilatory pathway that involves the fixation of carbon dioxide into citrulline has been established for the marine nitrogen fixing cyanobacterium *Anabaena* sp 1F (Tabita, 1987), and it has been suggested that the fixation of  $\text{CO}_2$  into citrulline may be related to the need for arginine in the synthesis of multi-L-arginine poly-L-aspartic acid (cyanophycin) granules (Weathers and Allen, 1978).

However, under conditions of active growth, the majority of the  $\text{CO}_2$  assimilated enters intermediary metabolism via the reaction catalysed by RuBisCO, which has been detected in all cyanobacteria investigated (Smith, 1982). Like other phototrophs, cyanobacteria possess metabolic mechanisms that permit survival during the regular dark periods they are exposed to in nature. During active growth, intermediates are removed from the Calvin cycle at various points for the synthesis of cell constituents, however from kinetic analysis of  $^{14}\text{C}$  flow, it was found that most of the  $\text{CO}_2$  assimilated was not incorporated equally into all growth polymers, but primarily a glucose containing polymer, glycogen (Pelroy and Bassham, 1972). The

reserve polymer cyanophycin, which is unique to cyanobacteria, has relatively little carbon from  $\text{CO}_2$  incorporated into it, and is apparently produced at the expense of cell protein (Allen et al., 1980).

Levi and Preiss (1976) have proposed that glycogen synthesis in cyanobacteria is regulated by the balance between phosphate and 3-phosphoglycerate, since the enzyme ADP-glucose pyrophosphorylase, which mediates synthesis of the monosaccharide donor units for polymerisation by glycogen synthetase, in a similar fashion to that of other oxygenic photosynthetic organisms, is inhibited by phosphate and stimulated by 3-phosphoglycerate. It has been demonstrated that cyanobacteria in the exponential phase of growth in batch photoautotrophic culture contain glycogen amounting to between 10-20% dry weight, and this can increase to 60% of dry weight for cultures starved of nitrogen (Allen and Smith, 1969). Interestingly, glucose added to nitrogen starved cultures of *Synechocystis* PCC6803, inhibited phycocyanin degradation, in a process which required metabolism of the sugar, and apparently irreversibly inactivated the phycocyaninase (Elmorjani and Herdman, 1987) suggesting a close link between the metabolism of the two polymers. Doolittle and Singer (1974) have shown that the photosynthetically obligate *Synechococcus* PCC6301 can maintain its viability over a 20 hr period under aerobic conditions in the dark without organic carbon, hence alternative energy yielding mechanisms must be present, so at the very minimum cell integrity and viability are maintained until the



next light period. The rate of ATP synthesis in the dark, without glucose, in *Anabaena cylindrica* was found by Bottomley and Stuart (1976) to be only 10% of that supported by photophosphorylation, however the fact that the cell maintained a high intracellular concentration of ATP indicated that demand for ATP in the dark was also much reduced, and has led Smith (1982) to postulate that this large pool of ATP is required for the maintenance of processes essential for the cyanobacterial cells survival, since all cyanobacteria exhibit a low rate of dark endogenous respiration (see Pearce and Carr, 1967).

The endogenous reserve role of cyanobacterial glycogen has been confirmed by workers incubating cyanobacteria in the dark under aerobic conditions, which results in a gradual decrease in glycogen content (see van Liere et al., 1979). As with the identification of the metabolic pathway of CO<sub>2</sub> fixation, the establishment of the oxidative pentose phosphate pathway as the principal metabolic route involved in the maintenance of energy levels in the dark also involved rapid radiolabelling studies (Pelroy and Bassham, 1972). The exclusive role of this pathway in cyanobacterial dark endogenous metabolism was established by the isolation of a mutant of the obligate autotroph *Synechococcus* PCC6301 by Doolittle and Singer (1974). This mutant died rapidly when placed in the dark, 90% of the population were non-viable within 1 hr, due to the absence of 6-phosphogluconate dehydrogenase. Phenotypic revertants selected for dark survival, were all double mutants, and had lost glucose-6-phosphate dehydrogenase in addition to

6-phosphogluconate dehydrogenase. These enzymes are involved in the oxidative pentose phosphate pathway, and are normally found at high levels in cyanobacteria grown under photoautotrophic conditions (Pearce and Carr, 1969; Pelroy et al., 1972). This double mutant did not consume detectable quantities of  $O_2$  in the dark, clearly implicating the enzymes in the dark endogenous metabolism of this organism (Doolittle and Singer, 1974). Since these two enzymes are NADP specific, it also confirmed the work of Biggins (1969), who as well as being the first person to demonstrate that ATP synthesis accompanied endogenous respiration, also suggested that NADP and not nicotinamide adenine dinucleotide (NAD) participated in respiratory electron transfer.

As can be seen from Fig. 1.1, the primary pathways of carbon metabolism in cyanobacteria in the light and dark are very similar, with several enzyme-catalysed reactions operative in both. Only two enzymes are specific to each pathway, phosphoribulokinase and RuBisCO in the light and glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the dark. This major change in metabolism that occurs depends on control mechanisms which prevent carbon dioxide fixation in the dark and block, under normal circumstances, the catabolism of endogenous polysaccharide reserves in the light. Pelroy et al. (1972) found that the enzymes mediating the reactions in Fig. 1.1 were synthesized under light and dark growth conditions, leading Smith (1982) to conclude that the control of the pathways operative in the light



and dark must be exerted at the enzymic level. Although the specific activity of RuBisCO has been shown to vary in chemostat cultures of *A.nidulans* (Karaguoni and Slater, 1979), in batch cultures only the specific activity of glucose-6-phosphate dehydrogenase has been shown to vary with growth conditions, it's activity often increasing considerably during dark growth (Pelroy et al., 1972). As the first enzyme of the oxidative pathway, glucose-6-phosphate dehydrogenase is a primary target for the suppression of respiration in the light, and the immediate cessation of CO<sub>2</sub> fixation on transfer from light to dark, and the appearance of a 6-phosphogluconate pool in <sup>14</sup>C and <sup>32</sup>P steady state labelling experiments (Pelroy and Bassham, 1972; Pelroy et al., 1976a,b), would seem to confirm this. The work of Doolittle and Singer (1974) also supports this view, since the mutant of *Synechococcus* 6301 which had lost 6-phosphogluconate dehydrogenase activity, was incapable of dark growth due to the rapid turn on of glucose-6-phosphate dehydrogenase, flooding the cells with 6-phosphogluconate, an unmetabolizable product in this mutant. Work with crude cell-free extracts of glucose-6-phosphate dehydrogenase from some unicellular cyanobacteria, showed it was specifically inhibited by ribulose-1,5-diphosphate (RUBP) (Pelroy and Bassham, 1972; Pelroy et al., 1976a). In partially purified samples from *Synechococcus* 6301 (Grossman and McGowan, 1975) and *Anabaena* 7120 (Schaeffer and Stanier, 1978) catalytic activity was inhibited by NADPH and ATP, however RUBP had no effect. In crude extracts of *Plectonema boryanum*, the activity of the

enzyme was not inhibited by RUBP, ATP or NADPH (Raboy et al., 1976) and so although some of the effects on enzyme activity fit in with the regulation of this enzyme and the pentose phosphate pathway, conclusive evidence is still required.

6-phosphogluconate, has in a number of studies been shown to reduce the catalytic activity of RuBisCO in crude enzyme extracts (Tabita and McFadden, 1972), permeabilized cells (Tabita and Colletti, 1979) and also the purified enzyme (Codd and Stewart, 1977). Pelroy et al. (1976a) however think the appearance of a 6-phosphogluconate pool is of little importance in the shut-down of  $\text{CO}_2$  fixation, since the concomittant decline in the RUBP concentration on transfer to the dark suggests RuBisCO is still functioning for the short time substrate is available and they suggest it is likely to be activated by the loss of non-cyclic electron flow. The energy dependence of the  $\text{C}_i$ -concentrating mechanism may also help explain this shut-down on transfer to the dark (see Section 1.5).

In principle, the oxidation of glucose-6-phosphate through the oxidative pentose phosphate pathway could be coupled with a terminal oxidation of pyruvate through the tricarboxylic acid (TCA) cycle. However, cyanobacteria do not synthesize

$\alpha$ -ketoglutarate dehydrogenase (Smith et al., 1967) and hence the TCA cycle cannot function as a respiratory pathway.

Consequently, like many bacteria with incomplete TCA cycles, the remaining enzymes of the TCA cycle have biosynthetic roles, such as the formation of amino acids from  $\alpha$ -ketoglutarate (Smith et al., 1967).

### 1.2.2 Heterotrophic growth and metabolism

Some cyanobacteria can, as well as growth on  $\text{CO}_2$  in the light, grow at the expense of a limited range of organic substrates. Such growth can take two different forms, photoheterotrophy, growth in the light on an organic substrate in the absence of net  $\text{CO}_2$  fixation, and chemoheterotrophy, growth on an organic substrate in complete darkness, the organic compound supplying the organism with a source of carbon and energy (Smith, 1982). These are distinct from mixotrophic growth, in which  $\text{CO}_2$  and organic substrate are assimilated simultaneously in amounts which vary with the culture conditions (Smith, 1973).

Photoheterotrophy has been demonstrated using several procedures which eliminate net  $\text{CO}_2$  fixation. These include excluding  $\text{CO}_2$  from the growth medium (Ingram et al., 1973), blocking its assimilation indirectly with the herbicide

3'-(3,4-dichlorophenyl) 1',1'-dimethylurea (DCMU) which inhibits non-cyclic electron flow from photosystem II (PSII) and thus NADPH production (Rippka, 1972) and lowering the incident light intensity to a point where net  $\text{CO}_2$  fixation is greatly reduced. (Van Baalen et al., 1971). Since the rate of ATP synthesis by cyclic photophosphorylation in the presence of DCMU is greater than that of respiratory ATP synthesis at the expense of an organic substrate in the dark, photoheterotrophic growth in the presence of DCMU is faster than dark growth at the expense of the same substrate (Rippka, 1972), making it the most convenient way to demonstrate photoheterotrophic growth.



Rippka et al. (1979) screened a wide variety of strains for photoheterotrophic growth using DCMU. Of 140 strains tested, just over half grew with an exogenous organic compound as carbon source, however this ability was not distributed evenly among the taxonomic groups, being far commoner amongst the filamentous strains, although some unicellular strains are capable of photoheterotrophic growth, including the genus *Synechocystis* (also see Rippka, 1972; Astier et al., 1984; Jansson et al., 1987). D-glucose, as seen in earlier studies (see Pearce and Carr, 1969; Pelroy et al., 1972) was the most common substrate metabolized, although growth was also seen by a few isolates on sucrose, D-fructose, D-ribose and glycerol.

In most cyanobacteria studied, heterotrophic growth is constitutive, occurring without a significant lag on transfer from autotrophic to heterotrophic conditions (see Beauclerk and Smith, 1978; Flores and Schmetterer, 1986) although in *Plectonema boryanum* it appears to be substrate inducible (Raboy and Padan, 1978).

Since the observation that *Aphanocapsa* PCC6714 was capable of heterotrophic growth at the expense of glucose (Rippka, 1972), a number of studies with this organism have firmly established the oxidative pentose phosphate pathway as the route for the aerobic metabolism of exogenous glucose (see Pelroy et al, 1972; Beauclerk and Smith, 1978; Joset-Espardellier et al., 1978). In fact with the exception of the unconfirmed work on growth of *Chlorogloeopsis* PCC6912 and *Nostoc* sp. MAC on acetate (Miller



and Allen, 1972; Ingram et al., 1973) all of the compounds that support the heterotrophic growth of cyanobacteria can be metabolized via this pathway. Fermentation and anaerobic respiration do not, with the exception of those cyanobacteria capable of anoxygenic photosynthesis (see Oren and Shilo, 1979; Padan and Cohen, 1982), contribute to the generation of energy in the dark in heterotrophic cyanobacteria.

Pelroy et al. (1972) reported that the relatively high rate of  $\text{CO}_2$  production from exogenous glucose and the saturation kinetics of this catabolic process by *Aphanocapsa* PCC6714 in comparison to other cyanobacteria was probably due to a transport mechanism for exogenous glucose in this organism. Using structural analogues of D-glucose which are not metabolized by the cell, it was found in *Nostoc* sp. MAC and *Aphanocapsa* 6714 (Beauclerk and Smith, 1978) and *Plectonema boryanum* (Raboy and Padan, 1978) that the analogue of glucose competitively inhibited the uptake of D-glucose, and in addition the unmodified analogue accumulated within the cell at 10-100 fold concentration. From these results it was suggested that there was a mechanism in operation for the active transport of D-glucose into the cell, using energy, under photoheterotrophic conditions, supplied by cyclic photophosphorylation. A number of other heterotrophic strains also show saturation kinetics in the uptake of organic compounds including *Plectonema boryanum* (sucrose); *Anabaena* PCC29413 (D-fructose) and *Chlorogloeopsis* PCC6912 (D-glucose) (More et al., 1979).

Prokaryotes have evolved several mechanisms for the transport of

solutes, which may be in equilibrium across a biological membrane, or concentrated on one side. There are five carrier mediated transport systems which occur in prokaryotes, and these are schematically represented in Fig. 1.2. Apart from facilitated diffusion, which is not coupled to metabolic energy, and is therefore not capable of accumulating a substance against a concentration gradient, the other systems are termed active or concentrative transport systems. For a full review of these mechanisms of solute transport see the review by Dills et al. (1980).

As already mentioned, much of the early work on heterotrophy in cyanobacteria utilized *Synechocystis* PCC6714 (formerly called *Aphanocapsa* 6714). Using *Synechocystis* PCC6803, a close relative of *Synechocystis* PCC6714 (Rippka et al., 1979) and capable of heterotrophic growth (Astier et al., 1984) Flores and Schmetterer (1986) found fructose (10 mM) inhibited growth. Fructose was found to be bactericidal for *Synechocystis* PCC6803, and ten independently isolated fructose resistant mutants each had an alteration in the glucose transport system, measured as uptake of glucose or its structural, non-metabolizable analogue 3-O-methyl-D-glucose (OMG). This suggested that fructose resistance may have resulted from a mutation in the glucose permease, the bactericidal effect produced from either an interaction of fructose with the permease, or as was thought more likely, transport of the bactericidal substance by the permease. The interaction of fructose and glucose is common in those organisms with a phosphoenolpyruvate sugar

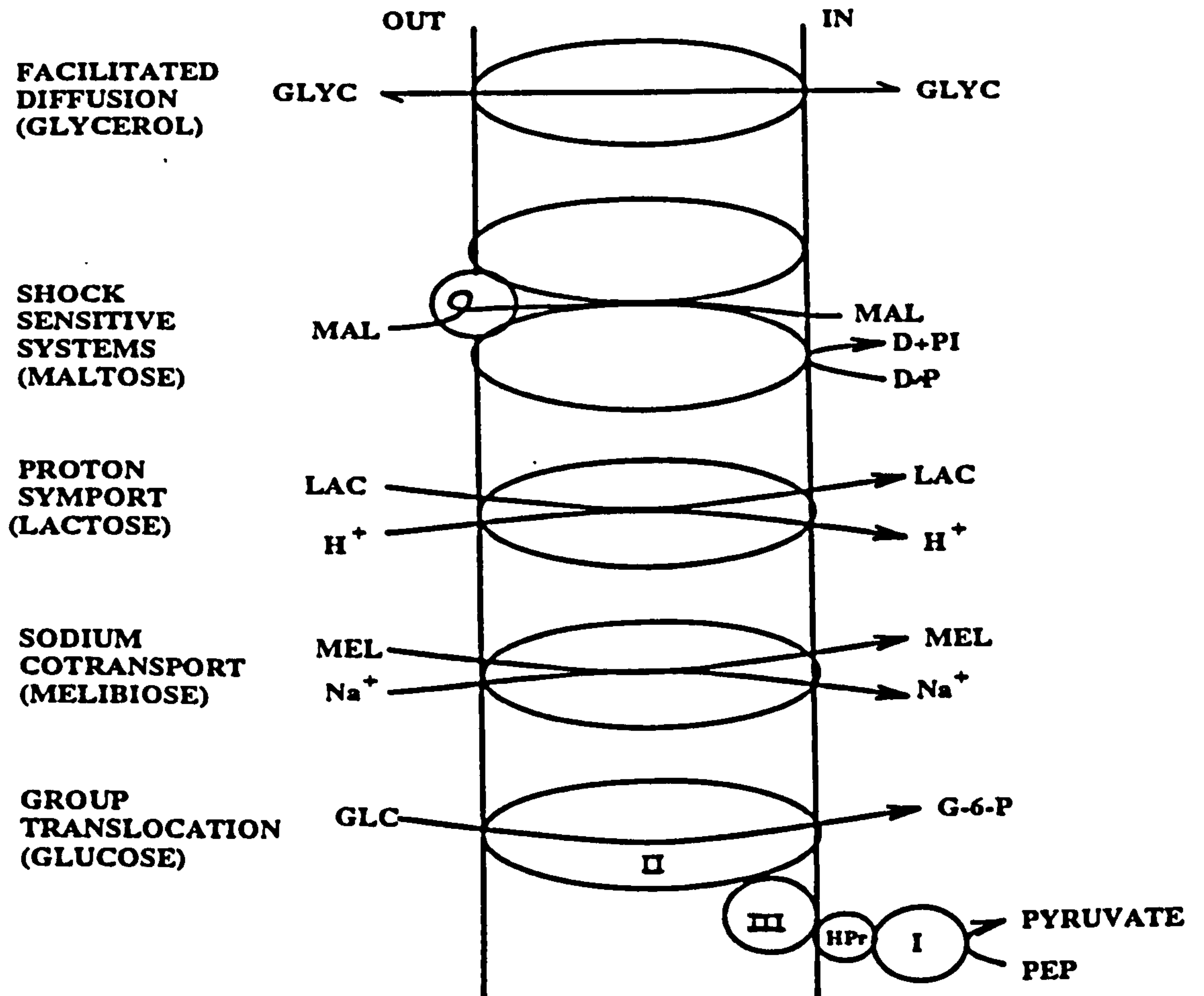


Figure 1.2. Carrier-mediated transport mechanisms. Representative examples (in parentheses) occur in *E. coli*. Abbreviations: GLYC, glycerol; MAL, maltose; LAC, lactose; MEL, melibiose; GLC, glucose; G-6-P, glucose 6-phosphate; PEP, phosphoenolpyruvate; D + Pi, nonphosphorylated donor plus inorganic phosphate; D-P, high energy phosphorylated donor; I, enzyme I; II, enzyme II; III, enzyme III, (from Dills *et al*, 1980).



phosphotransferase system (PTS) (Dills et al., 1980), however no evidence of a PTS was found in *Aphanocapsa* PCC6714 (Beauclerk and Smith, 1978) or *Plectonema boryanum* (Raboy and Padan, 1978). Joset et al. (1988) further characterized the physiological requirements and genetics of the glucose-fructose permease in *Synechocystis* PCC6803 and also in *Synechocystis* PCC6714. They obtained two classes of fructose resistant mutants, group A were partially, and group B totally deficient for glucose transport. They found that rates of fructose transport in wild-type (WT) cells indicated a low affinity for fructose of the glucose permease, however in competition experiments, a major proportion of fructose was taken up through the glucose permease. The unicity for glucose and fructose transport in these organisms was demonstrated by the isolation of spontaneous mutants in *Synechocystis* PCC6714 and *Synechocystis* PCC6803 showing the pleiotropic phenotype,  $\text{Glu}^-$ ,  $\text{Fru}^R$ ,  $\text{transport}^-$ , and in *Synechocystis* PCC6803 by the transformation of a  $\text{Fru}^R$  mutant with wild-type DNA, which resulted in transformants with a  $\text{Glu}^+$ ,  $\text{Fru}^R$  phenotype. Using the mutant  $\text{Fru}^R$  B5, Zhang et al. (1989) performed complementation studies, which enabled the corresponding chromosomal gene, *glcP* to be cloned. The protein predicted was 468 residues long, with a calculated molecular weight of 49,743, and as such was the first sugar transport system to be analysed at the molecular level in cyanobacteria. It was found to be analogous to a group of homologous sugar transporters using non-phosphorylating processes from both eukaryotes and other prokaryotes. Schmetterer (1990), working



with *Synechocystis* PCC6803 has independently sequenced the glucose transporter gene from this organism, and like Zhang et al (1989) has found that it has a highly conserved sequence when compared with mammalian glucose transporters.

Beauclerk and Smith (1978) suggested that the absence of the sugar transport mechanism was the reason other strains failed to grow heterotrophically, since they possess all of the requisite enzymes within the cell. Doolittle (1979) thought that impermeability to potential growth substrates is unlikely to be the only barrier to heterotrophic growth. He proposed that the metabolic priority of cyanobacteria in the dark is to restrict carbohydrate metabolism in order to ensure survival through the period of darkness, and heterotrophic cyanobacteria can distinguish between conditions where an exogenous growth material is available from one in which they are totally dependent on their endogenous reserves. F.Joset (personal communication) has transferred the *glcP* gene into *Synechococcus* PCC7942 on a plasmid, and has found that this alters the phenotype of this organism from  $\text{Glu}^-$  to  $\text{Glu}^+$ , adding substance to Beauclerk and Smith's hypothesis (1978) that heterotrophic growth is dependent solely upon possession of an active transport permease.

### 1.2.3 Photorespiration

The inhibition of photosynthetic  $\text{CO}_2$  assimilation by oxygen (Glover and Morris, 1981) and the formation and excretion of glycollate (Codd et al., 1976) in cyanobacteria, has been shown

to be at least partly due to the competitive inhibition of the carboxylase reaction of RuBisCO by oxygen (see Codd and Stewart, 1977; Andrews and Abel, 1981).

The oxygenation of RUBP results in the formation of one molecule each of 3-phosphoglycerate and 2-phosphoglycollate (Miziorko and Lorimer, 1983). The 2-phosphoglycollate is converted to glycollate. If glycollate production rates exceed the capacity for oxidation by glycollate dehydrogenase, or if this enzyme is inhibited or repressed, glycollate excretion will occur (Codd et al., 1976; King and Anderson, 1980). Alternatively, the glycollate produced can be metabolized through the photorespiratory or C-2 pathway (see Fig. 1.3). The glycollate formed can be recycled to the central pool via glycine and serine (Dohler and Przybylla, 1973 as cited by Tabita, 1987) or via tartronic semialdehyde (Codd and Stewart, 1973). Although exogenous glycollate can be assimilated by cyanobacteria (Codd and Stewart, 1973), it cannot support chemoheterotrophic (Codd and Stewart, 1974) or photoheterotrophic growth (Stanier and Cohen-Bazire, 1977) of these organisms.

Every RuBisCO isolated to date, including the enzyme from anaerobic bacteria, is capable of catalysing the oxygenase reaction (Tabita, 1987), suggesting that the statement by Lorimer and Andrews (1973) that "the oxygenase function of RuBisCO is an inherent property of the enzyme and proceeds as a consequence of the enzymatic formation of the ene-diol of RUBP", is correct.

The oxygenase activity of RuBisCO (photorespiration) is regarded

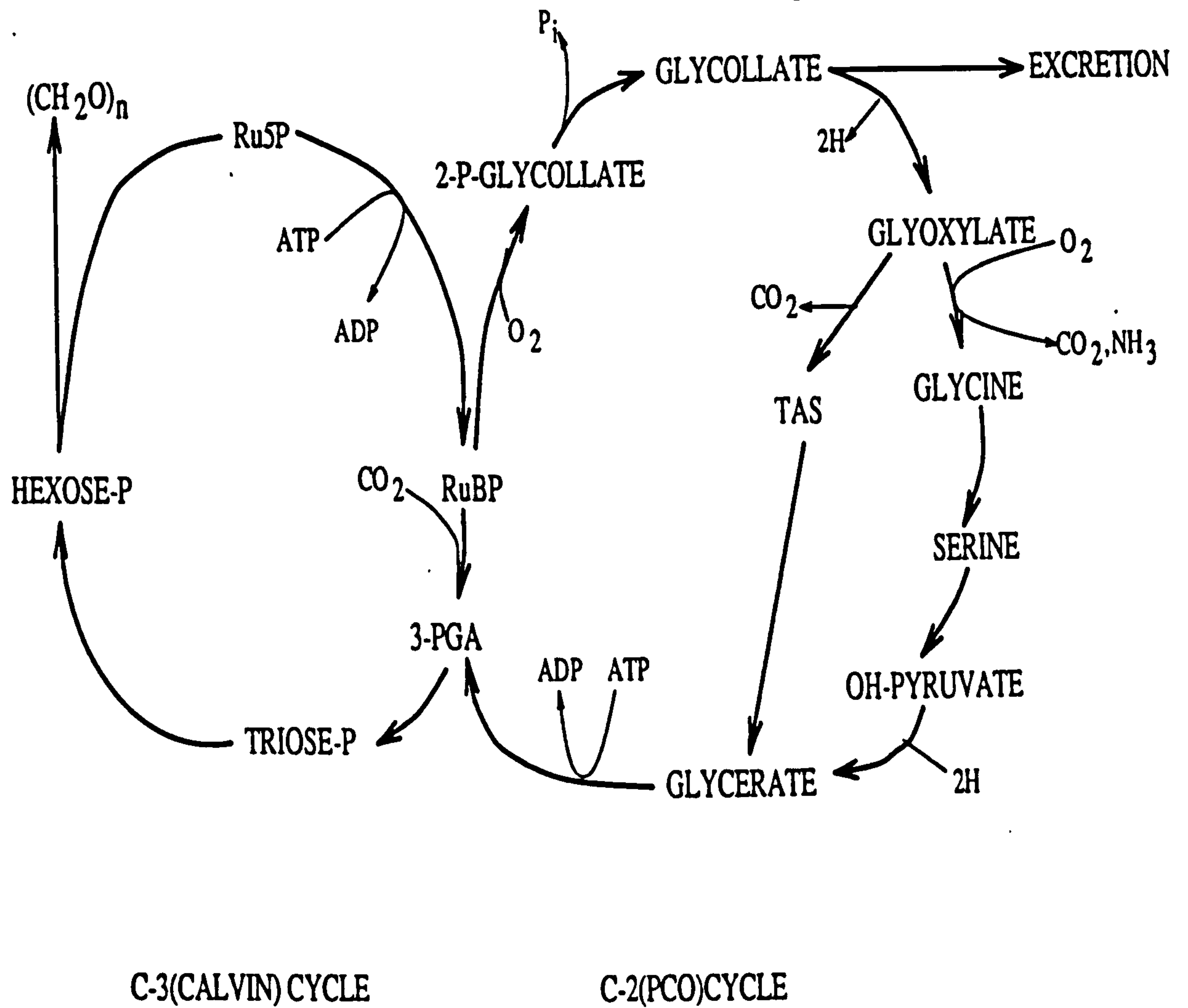


Figure 1.3. The Photorespiratory or C<sub>2</sub> pathway showing its integration with the Calvin cycle. (From Codd, 1984).  
TAS = Tartronic Semialdehyde



by many plant physiologists as the major constraint on plant productivity, and helps to explain why terrestrial  $C_3$  plants possess RuBisCO in such abundance, in some cases accounting for more than 65% of total soluble protein (Ellis 1979).

Some plants however do not photorespire under normal growth conditions, due to an accessory pathway for transporting and concentrating carbon dioxide at the site of the calvin cycle in their photosynthetic cells. These are the  $C_4$  plants. At the high ambient light intensities usually experienced by these plants, photosynthetic rates are limited by the availability of carbon dioxide (Wareing et al., 1968). Atmospheric carbon dioxide is initially fixed by phosphoenolpyruvate (PEP) carboxylase into  $C_4$  acids, within mesophyll cells. These acids are then transported to the bundle-sheath where the acids are decarboxylated and the carbon dioxide released refixed by RuBisCO (Figure 1.4). PEP carboxylation and  $C_4$  acid degradation both exceed the rate of RUBP carboxylation; the carbon dioxide concentration around RuBisCO is maintained above ambient and photorespiration reduced (Hatch, 1971).

A similar phenomenon is seen in cyanobacteria and green algae. Kaplan and Berry (1981) found that in low  $CO_2$ -grown cells of *Chlamydomonas reinhardtii*, glycollate excretion only occurred at external  $CO_2$  concentrations much lower than those necessary to stimulate glycollate excretion in high  $CO_2$ -grown cells. Other workers found the oxygenase activity of RuBisCO completely suppressed in low  $CO_2$ -grown organisms (Lloyd et al., 1977;



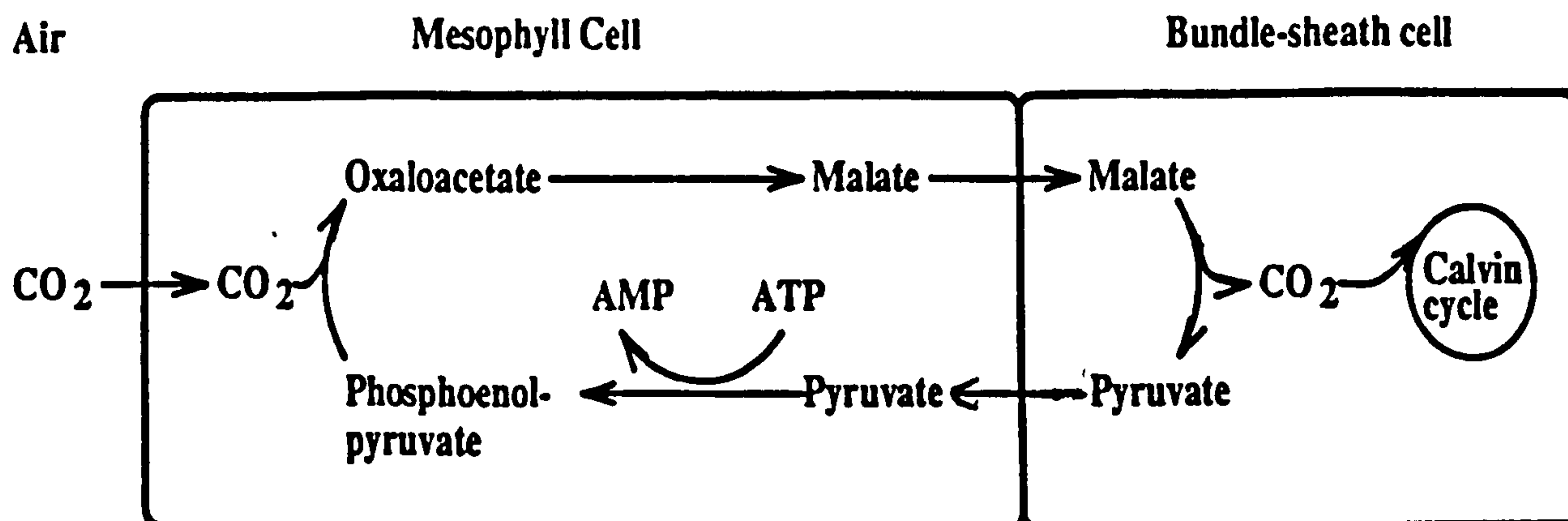


Figure 1.4. Schematic diagram of the C4 pathway.

Birmingham et al, 1982). The suppression of photorespiration in low  $\text{CO}_2$ -grown cells of cyanobacteria is a consequence of the inorganic carbon (Ci) concentrating mechanism these organisms possess (see Badger, 1987 and section 1.5) .

### 1.3 The acquisition of carbon by phototrophic organisms

The acquisition of carbon dioxide from the external environment and its supply as a substrate for RuBisCO is of great significance, as regards the efficient operation of photosynthesis.

The rate of photosynthesis, taken as  $\text{CO}_2$  fixation, under ambient conditions in terrestrial  $\text{C}_3$  plants is primarily determined by the affinity of RuBisCO for carbon dioxide and oxygen (Farquhar et al., 1980). Little or no biochemical action is thought to precede the diffusion of carbon dioxide to the active site of RuBisCO (Ogren, 1984) since the effective aqueous diffusion path has been reduced to a few micrometers (Raven, 1984). The efficiency of fixation and affinity of RuBisCO for  $\text{CO}_2$  are surprisingly poor (Andrews and Lorimer, 1987) and since oxygen competes with carbon dioxide at this catalytic site maximum photosynthetic rates cannot be achieved (Ogren 1984).

This contrasts with the capture of carbon dioxide in the aquatic environment, which is where the majority of cyanobacteria reside.

The concentration of carbon dioxide in air is 0.03-0.04% (v/v). Under conditions where it is equilibrated with air, the concentration of dissolved carbon dioxide in fresh or marine

water is 10-12  $\mu\text{M}$  at 25°C. Problems arise in obtaining carbon dioxide from an aquatic environment largely as a result of the physical chemistry relating to inorganic carbon (Ci) species in solution. Inorganic carbon exists in solution as three major forms (see Figure 1.5). All of these forms have a slow diffusion rate, that of carbon dioxide being some  $10^4$  lower in water than air. As can be seen from figure 1.5 the chemical equilibrium between the species is mainly influenced by the pH of the medium, and to a lesser extent by the temperature and salinity. In acidic conditions  $\text{CO}_2$  is the main constituent, and there is an increasing predominance of the  $\text{HCO}_3^-$  ion above pH 6, and then  $\text{CO}_3^{2-}$  above pH 9.

If the aqueous medium is in equilibrium with the gas phase the carbon dioxide in solution will remain constant regardless of pH, however the total dissolved inorganic carbon (DIC) will increase at elevated pH. The pH of seawater, which accounts for the greatest proportion of natural waters is 7.8-8.3. The concentration of DIC in the open sea is around 2mM, with the concentration of  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$  and  $\text{CO}_2$  being 1.8, 0.35 and 0.01-0.02 mM respectively (Round, 1981). Freshwater environments are, unlike seawater, poorly buffered, and photosynthetic activity will lead to the pH, of what in most cases are alkaline environments, increasing and consequently causing a further diminution of the  $\text{CO}_2$  supply. Thus Talling (1976) observed that in two British lakes of low alkalinity, the pH values rose to greater than 10 and the  $\text{CO}_2$  concentration dropped to as low as 0.5  $\mu\text{M}$ . Therefore depending on the pH of the water little of the

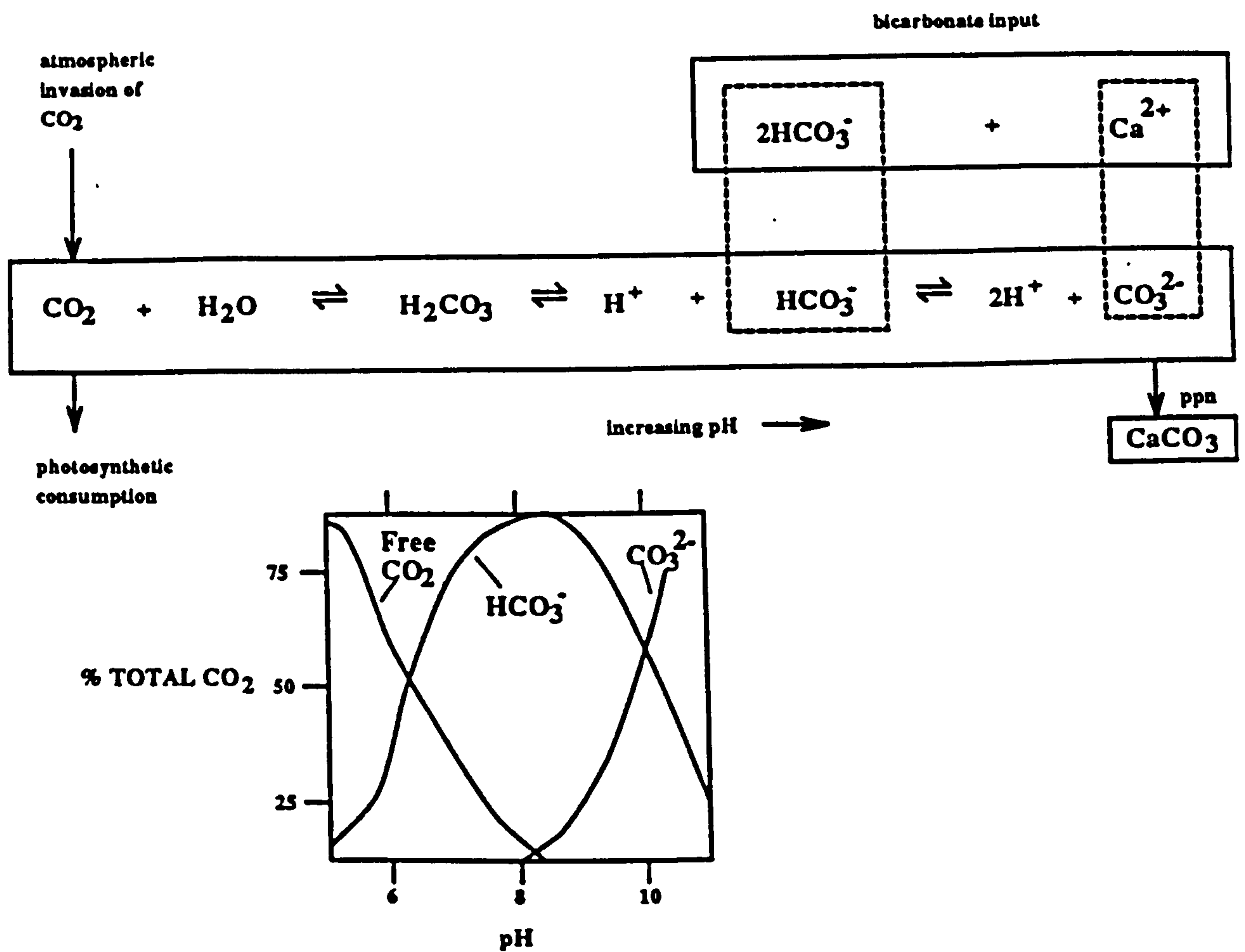


Figure 1.5. The pH-carbon dioxide-bicarbonate system in freshwaters. (From Round, 1981).



Ci in the environment may be present as  $\text{CO}_2$  and the  $\text{CO}_2/\text{O}_2$  ratio unfavourable for photosynthesis (Ogren, 1984).

Supply of Ci to aquatic phototrophs however is not limited by the diffusion of  $\text{CO}_2$ , as the diffusion of  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  and their conversion to  $\text{CO}_2$  can also contribute, and since concentrations of bicarbonate are often several orders of magnitude higher than of  $\text{CO}_2$ , this represents a potentially major pathway for the supply of  $\text{CO}_2$ . However the interconversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  is slow, 60s being the time for complete equilibrium between the species at  $25^\circ\text{C}$  at pH7 (Lehman, 1978), a figure which increases with increasing pH. This can limit  $\text{CO}_2$  supply, particularly if photosynthesis is rapid. Detailed analysis of the kinetic limitations to  $\text{CO}_2$  supply by diffusion and interconversion of Ci species have been made by Lehman (1978) and Miller (1985).

In spite of these physical and chemical complications regarding the capture of inorganic carbon, cyanobacteria and other aquatic phototrophs grow over a wide range of DIC concentrations and flourish at concentrations of  $\text{CO}_2$  where direct fixation by RuBisCO would be inconsequential (Jensen and Bahr, 1977). In addition there is little, if any effect of oxygen concentration on  $\text{CO}_2$  fixation, the oxygenase activity of RuBisCO being suppressed (Andrews and Lorimer, 1987).

This atypical behaviour relative to terrestrial  $\text{C}_3$  plants is not due to an alternative pathway for  $\text{CO}_2$  fixation, as seen in the  $\text{C}_4$  plants, nor is it due to an increased affinity for  $\text{CO}_2$  by the

RuBisCO of aquatic phototrophs. In fact the  $K_m$  of RuBisCO for  $CO_2$  is higher than that of many terrestrial  $C_3$  plants, values of 250  $\mu M$  being reported for the cyanobacterium *Synechococcus sp.* (Andrews and Abel, 1981), more than two-fold higher than the  $CO_2$  concentration in air equilibrated solutions.

These characteristics are due to the major strategy that has evolved in many aquatic phototrophs, of a system for the active transport of  $C_i$  into cells. This transport results in the accumulation of  $C_i$  within the cell above the external concentration and the establishment from this of an elevated internal  $C_i$  concentration, hence the term "  $C_i$  concentrating mechanism " (Badger et al, 1978).

This mechanism utilises both  $HCO_3^-$  and  $CO_2$  as substrates from the external medium, operating as a " front end " to photosynthesis, modifying the  $C_3$  characteristics of the organisms as discussed above (see reviews by Badger, 1987; Miller, 1990; Kaplan et al., 1991).

#### 1.4 Historical perspectives of inorganic carbon transport

The recognition of the ecological significance of  $HCO_3^-$  as a carbon source in natural waters, was first discovered around the turn of the century, by amongst others Birge (1907) who stated " It appears that the algae .... can obtain their supply of carbon from the carbon dioxide of the bicarbonate dissolved in the water. This fact introduces a wholly new feature into the story of the food supply of the plants. It provides a chemical carrier

for the carbon dioxide, which may carry this gas somewhat as the haemoglobin carries oxygen in the blood ".

Arens (1933) showed in more detailed studies that the calcification of the leaves of aquatic angiosperms, by now a widely recognised phenomenon, was due to light-dependent pH changes promoting calcification and starch deposition on the upper leaf surface and bicarbonate uptake on the lower leaf surface. Steemann-Nielsen (1947) using a more sophisticated experimental procedure involving measurements of oxygen evolution, showed that both leaf surfaces and not just the lower one as previously thought could absorb  $\text{HCO}_3^-$ . The transport of  $\text{HCO}_3^-$  was interpreted as a simple uniport, involving a specific energy-dependent carrier, Raven's review in 1970 summing up the thoughts on  $\text{HCO}_3^-$  uptake by aquatic organisms at the time. It was not until 1976, that it was clearly established that  $\text{HCO}_3^-$  transport could act as a photosynthetic  $\text{CO}_2$  concentrating mechanism. Berry et al. (1976) found that depending on the  $\text{CO}_2$  concentration used for growth, cells of *Chlamydomonas reinhardtii* showed differing photosynthetic responses. Cells grown in high  $\text{CO}_2$  conditions (5%  $\text{CO}_2$ ) had a photosynthetic affinity for external  $\text{CO}_2$  twenty times lower than low  $\text{CO}_2$  (air grown) cells. In addition they exhibited a much greater sensitivity to  $\text{O}_2$  concentrations during photosynthesis, as measured by impaired growth rates and glycollate excretion with increasing  $\text{O}_2$  concentration. Low  $\text{CO}_2$  grown cells exhibited similar characteristics to those seen in  $\text{C}_4$  plants. Findenegg (1976), working with the green algae *Scenedesmus obliquus*, found



similar results, namely a twenty fold higher photosynthetic affinity for external  $\text{CO}_2$  in low  $\text{CO}_2$  as opposed to high  $\text{CO}_2$  grown cells, and the same trend was seen with *Chlorella vulgaris* 11h (Hogetsu and Miyachi, 1977). This idea of different physiological states existing when organisms are grown under different  $\text{CO}_2$  regimes was not new in itself, Briggs and Whittingham in their 1952 review acknowledging the fact that *Chlorella* exhibited different physiological characteristics which depended on whether the cells were exposed to air (0.03%  $\text{CO}_2$ ) or air enriched with 1-5%  $\text{CO}_2$ . Another factor reported to vary depending on the  $\text{CO}_2$  regime of the cells during growth was the enzyme carbonic anhydrase. Raven (1970) stated that at high pH's carbonic anhydrase would be necessary for bicarbonate assimilation, and the activity of this enzyme was found to be higher in low as opposed to high  $\text{CO}_2$  grown cells by a number of workers using different aquatic organisms (Graham and Reed, 1971; Findenegg, 1976; Ingle and Coleman, 1976). Although this enzyme will promote the equilibrium between  $\text{HCO}_3^-$  and  $\text{CO}_2$ , Berry et al., (1976) realized that the overall affinity of the cells for  $\text{CO}_2$  would still be limited by the intrinsic properties of RuBisCO. Since low  $\text{CO}_2$  grown cells of *Chlamydomonas reinhardtii* had an affinity for  $\text{CO}_2$  an order of magnitude higher than the measured *in vitro* properties of RuBisCO, these cells may actively accumulate bicarbonate by a metabolic influx pump elevating the internal  $\text{CO}_2$  concentration above the external one. Werden and Heldt (1972) had shown bicarbonate accumulation in intact chloroplasts using a silicone layer filtering



centrifugation technique, and using this technique, Badger et al. (1978) first obtained evidence for active transport and accumulation of bicarbonate in air grown cells of *Chlamydomonas reinhardtii*. Badger et al. (1980) followed up the initial observation of active bicarbonate accumulation to show that low CO<sub>2</sub> grown cells of *Chlamydomonas reinhardtii* could concentrate CO<sub>2</sub> internally up to 40-fold higher than the external concentration, and that this accumulation was dependent upon an energy supply linked to photophosphorylation. Using similar techniques Kaplan et al. (1980) and Miller and Colman (1980) showed that the cyanobacteria *Anabaena variabilis* and *Coccochloris peniocyctis* had an even greater capacity to concentrate Ci, with an internal concentration over 1000 times greater than the external one, in air-grown cells when there was a low Ci concentration in the external medium. This ability to actively concentrate Ci internally up to several orders of magnitude above the external concentration when grown in air, as opposed to high CO<sub>2</sub> has also been reported in a variety of organisms including other cyanobacteria (Coleman and Colman, 1981; Kaplan, 1981; Badger and Andrews, 1982; Marcus et al, 1982; Shelp and Canvin, 1984; Volokita et al., 1984) green algae (Beardall, 1981; Beardall and Raven, 1981; Zenvirth and Kaplan, 1981; Spalding and Ogren, 1983; Tsuzuki et al., 1985) red algae (Smith and Bidwell, 1987) *Thiobacillus neapolitanus* (Holthuijzen et al., 1987) and even intact chloroplasts of *Chlamydomonas reinhardtii* (Moroney et al., 1987).

## 1.5. The inorganic carbon concentrating mechanism in cyanobacteria

### 1.5.1 Physiological aspects

The operation of a mechanism to concentrate  $\text{CO}_2$  in air-grown batch cultures of cyanobacteria was apparent from experimental evidence. The *in vitro* values measured for cyanobacterial RuBisCO  $K_m$ 's ranged between 200-330  $\mu\text{M}$  (Kaplan et al., 1980; Andrews and Abel, 1981), and the effective  $K_m$  ( $\text{CO}_2$ ) of RubisCO in the presence of  $\text{O}_2$  was even greater, about 600  $\mu\text{M}$  (Pierce and Omata, 1988).

However, the  $\text{CO}_2$  compensation point of cyanobacteria was very low and insensitive to oxygen (Birmingham and Colman, 1979), and there was no evidence of the oxygenase action of RuBisCO or photorespiration (Lloyd et al., 1977; Birmingham et al., 1982).

The apparent photosynthetic affinity of intact cells of cyanobacteria for carbon dioxide was found to be up to several orders of magnitude lower than that of the isolated enzyme. Values for batch grown cyanobacteria were found to range between 0.1-2.0  $\mu\text{M}$  (Kaplan et al., 1980; Miller and Colman, 1980; Kaplan, 1981; Badger and Andrews, 1982; Shelp and Calvin, 1984; Abe et al., 1987), whilst in bicarbonate-limited chemostats,  $K_m$  ( $\text{CO}_2$ ) values as low as 5.6-8.4 nM were obtained at pH 9.6 (Miller et al., 1984a).

Badger et al. (1978) were the first to prove the operation of a  $\text{CO}_2$ -concentrating mechanism. They found in *Anabaena variabilis* that  $\text{Ci}$  was accumulated internally in excess of 1000-fold over the extracellular concentration, whilst Kaplan et al. (1980)

using the same organism, found a 500 fold difference, and also found that the  $V_{max}$  for  $\text{Ci}$  transport of low  $\text{CO}_2$  grown cells was 10-fold higher than the  $V_{max}$  of photosynthesis. The majority of the work on the kinetics of  $\text{Ci}$  uptake has involved non steady-state conditions, where the time course of  $\text{Ci}$  accumulation is conducted under conditions where the internal  $\text{Ci}$  pool is depleted, the initial slope of  $\text{Ci}$  accumulation representing an estimate of the maximum rate at which the transport system can function. Using *Synechococcus* sp. Nageli under conditions where gross  $\text{Ci}$  uptake, gross photosynthesis and gross  $\text{Ci}$  leakage were all accounted for, Badger et al. (1985) found that gross  $\text{Ci}$  uptake did not exceed net photosynthesis by more than 30% when  $\text{CO}_2$  was just saturating. They concluded that although potential rates of  $\text{Ci}$  transport are very high, the steady state fluxes of  $\text{Ci}$  in photosynthesizing cells are regulated so as to be only sufficiently in excess of photosynthesis so internal  $\text{CO}_2$  levels are only just saturating for RuBisCO.

Miller and Colman (1980) proved conclusively that cyanobacteria could transport bicarbonate. They observed rates of  $\text{CO}_2$  fixation in *Coccochloris peniocyctis*, at pH 9.58, up to 50-fold higher than could be accounted for by interconversion between  $\text{CO}_2$  and  $\text{HCO}_3^-$  in the medium, hence  $\text{CO}_2$  fixation was being supported by a substantial bicarbonate influx, which represented active transport, since it was being accumulated against a concentration gradient. Most of the early work, carried out using the silicone oil centrifugation method (Section 3.3.3.1),



assumed that the mechanistic basis for the  $C_i$  concentrating mechanism utilized bicarbonate as the sole species for transport, since at the alkaline pH values favoured by most cyanobacteria, bicarbonate is the major source of  $C_i$  available to the concentrating mechanism (Badger et al., 1978; Badger et al., 1980; Kaplan et al., 1980; Coleman and Colman, 1981; Kaplan, 1981; Kaplan et al., 1982; Shelp and Calvin, 1984). Shiraiwa and Miyachi (1985) however, concluded that carbon dioxide is the active species of  $C_i$  utilized irrespective of external  $C_i$  conditions during growth.

Badger and Andrews (1982) using the techniques of silicone oil centrifugation and membrane inlet mass spectrometry (MIMS), were the first to suggest that both carbon dioxide and bicarbonate were capable of acting as substrate for a single complex mechanism in a marine *Synechococcus* sp.. MIMS involves incubating cells in a cuvette sealed from the atmosphere, but attached to a mass spectrometer via a capillary inlet whose end is covered by a dimethylsilicone membrane (gas permeable). Dissolved gases leak slowly across the membrane inlet and into the mass spectrometer allowing changes in the concentration of  $CO_2$  in the gas phase to be monitored. Badger and Andrews (1982) found that under non steady-state conditions, illumination of the cells at pH 8.2 led to such a rapid uptake of free  $CO_2$  that the extracellular concentration dropped to nearly zero. This could not be accounted for by  $CO_2$  fixation, and so represented active  $CO_2$  transport.  $HCO_3^-$  uptake was slower when given at an equivalent concentration to  $CO_2$ , however during isotope



disequilibrium experiments both species were taken up. Badger and Gallacher (1987) found similar results in *Synechococcus* PCC6301. Under steady state photosynthesis in seawater equilibrated with air however, Badger and Andrews (1982) found that  $\text{HCO}_3^-$  uptake into the cell is the primary source of internal Ci. It is now accepted that both bicarbonate and carbon dioxide can act as substrate for the inorganic carbon concentrating mechanism (see Miller et al., 1988a; and reviews by Badger, 1987; Miller, 1990 and Miller et al., 1990).

There is however still controversy as to whether a single common transport system or separate transport systems operate for these two Ci species. A differential  $\text{Na}^+$  requirement for  $\text{CO}_2$  and bicarbonate transport has led some authors to suggest that two separate systems exist (Espie et al., 1988a), whilst on the basis of ethoxymolamide inhibition of Ci transport it has been proposed that the dehydration of bicarbonate and rehydration of  $\text{CO}_2$  are successive steps, suggesting a common transport mechanism (Badger, 1987).

It has long been known that high  $\text{CO}_2$ -grown cells accumulate Ci internally, but the rate of accumulation and the steady state Ci concentration reached internally are much lower than in low  $\text{CO}_2$ -grown cells (Kaplan et al., 1980). This has been attributed to the fact that cells grown on high levels of  $\text{CO}_2$  lose their capacity for  $\text{HCO}_3^-$  transport, whilst retaining their capacity for active  $\text{CO}_2$  transport (Abe et al., 1987a; Badger and Gallacher, 1987; Miller and Calvin, 1987; Schwarz et al., 1988; Price and Badger, 1989a), although this  $\text{CO}_2$  transport had a 5-10

fold lower affinity for  $\text{CO}_2$  than seen in air grown cells (Miller and Calvin, 1987; Price and Badger, 1989b).

It was shown by Miller et al. (1984a), working with chemostat cultures of *Synechococcus* UTEX625 that it was not only high  $\text{CO}_2$ , but rather high DIC concentrations during growth that gave rise to cells with a low affinity for  $\text{Ci}$ . At pH 9.2 the  $\text{CO}_2$  concentration was very low, 2.3  $\mu\text{M}$  and yet the cells had characteristics of high  $\text{CO}_2$ -grown cells. Mayo et al. (1986 & 1989) and Badger and Gallacher (1987) have also shown that it is total DIC concentration during growth that determines the affinity of the cells for  $\text{Ci}$ . These authors have also proven the prediction of Turpin et al. (1985), that at intermediate DIC concentrations in batch culture, between 40 and 1500  $\mu\text{M}$ , a continuum of cell types with intermediate characteristics between fully adapted high and low  $\text{Ci}$  cells should be found. As these changes in  $K_{0.5}\text{DIC}$  occur in response to DIC concentrations commonly found in natural systems, Mayo et al. (1986) have suggested this adaptation might be of ecological significance, with the affinity of the  $\text{Ci}$  transport mechanism being adjustable so that the  $K_{0.5}\text{DIC}$  is close to the DIC concentration of the growth medium.

### 1.5.2 Inhibitor studies

A number of details about the characteristics of the  $\text{Ci}$  concentrating system have been elucidated through the use of inhibitors to "knock out" various components of the system. It was initially hypothesized that the  $\text{Ci}$  concentrating

mechanism was linked to photosynthesis through the synthesis of ATP. The inhibition of  $C_i$  transport by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a photosynthetic electron transport inhibitor, supported this hypothesis (see Kaplan et al., 1980; Miller and Colman, 1980; Shelp and Calvin, 1984). Further work by Ogawa et al. (1985), using infra red gas analysis (IRGA) to monitor the post illumination  $CO_2$  burst coupled with inhibitor studies in *Anacystis nidulans*, has however contradicted this hypothesis. They found that the  $CO_2$  burst was insensitive to DCMU in the presence of iodoacetamide, which knocked out  $CO_2$  fixation. In the absence of iodoacetamide DCMU inhibited the  $CO_2$  burst. Thus in the presence of iodoacetamide  $C_i$  transport did not require linear electron flow and was driven only by PSI cyclic electron flow. They ascribed the inhibitory effect of DCMU in the absence of iodoacetamide to the inhibition of NADP reduction, keeping the ratio of NADPH:NADP low, and thereby inhibiting cyclic electron flow. They also found that the  $CO_2$  burst was inhibited by draining electrons from ferredoxin, and hence PSI, (even when ATP was still produced) and by addition of either the ATPase inhibitor dicyclohexylcarbodiimide (DCCD) or the uncoupler carbonylcyanidechlorophenylhydrazone (CCCP). They concluded from these results that both cyclic electron flow and ATP are required to drive  $C_i$  transport. Interestingly, Manodori and Melis (1984) found that low  $CO_2$ -grown cells of the same organism had greater relative numbers of PSI reaction centres than those of high  $CO_2$ -grown cells, which they theorized might facilitate



greater rates of ATP synthesis via cyclic electron flow.

A number of other workers have also used inhibitors of CO<sub>2</sub> fixation and ATP synthesis to demonstrate certain features of the Ci concentrating mechanism in a particular organism, amongst them Miller et al. (1988b), who using *Synechococcus* UTEX625 found that CO<sub>2</sub> transport continued in the absence of CO<sub>2</sub> fixation against a concentration gradient of 18,000:1, and was completely inhibited by the ATPase inhibitor diethylstilbestrol.

The observation that carbonic anhydrase (CA) activity in algal cells is negatively correlated with glycollate excretion (Ingle and Colman, 1976) and positively correlated with photosynthetic rate under low CO<sub>2</sub> conditions (Reed and Graham, 1977), has led many workers to search for its activity to help explain their proposed models of Ci transport (see Volokita et al., 1984; Price and Badger, 1989a). The activity of carbonic anhydrase prove difficult to establish in cyanobacteria in early studies (Kaplan et al., 1980; Volokita et al., 1984), although the use of the mass spectrometer has significantly improved detection of the very low levels of CA activity observed in cyanobacteria in comparison with green algae, so that its occurrence is now firmly established (see Miller et al., 1990). In the early 1980's the indirect approach of studying the effect of CA inhibitors on Ci transport and accumulation, allowed workers to establish that Ci uptake was inhibited by the CA inhibitor ethoxycarbonyl amide (EZ), a lipid soluble CA inhibitor, leading Volokita et al. (1984) to include a "CA-like moiety" in their proposed transport mechanism



(see Fig. 1.7 A). Volokita et al. (1984), and Abe et al. (1987c), both using *A. variabilis* also found that Ci uptake was sensitive to ethoxycarbonyl amide when  $\text{CO}_2$  was used as substrate for the transport mechanism. Spiller et al. (1988) performed inhibitor studies on the  $^{18}\text{O}$  exchange kinetics of *Synechococcus* UTEX2380, from which they concluded the role of PSI was to provide energy for the active uptake of Ci into the cell, where CA catalyses the interconversion between  $\text{HCO}_3^-$  and  $\text{CO}_2$ . Price and Badger (1989a,b) found that in *Synechococcus* PCC7942, EZ inhibited the rate of  $\text{CO}_2$  uptake and reduced the size of the internal Ci pool in high Ci grown cells (1%  $\text{CO}_2$  [v/v]), whilst in low Ci grown cells, it inhibited both  $\text{CO}_2$  and  $\text{HCO}_3^-$  uptake, suggesting that transport of the two Ci species occurred via a single transport mechanism. Water soluble CA inhibitors (methazolamide and acetazolamide) were found to have little, if any effect on  $\text{CO}_2$  usage, and EZ had no apparent effect on internal  $\text{CO}_2/\text{HCO}_3^-$  equilibrium, leading these workers to propose a model of Ci uptake utilizing a membrane bound CA activity (Price and Badger, 1989a,b). Acetazolamide did however inhibit CA activity in *Synechocystis* PCC6714 both *in vivo* and *in vitro* (Bedu et al., 1989).

It has been possible to study  $\text{CO}_2$  transport in isolation in *A. variabilis* and *Synechococcus* UTEX625 because these organisms can be grown so that mM concentrations of  $\text{Na}^+$  are needed for  $\text{HCO}_3^-$  transport (Kaplan et al., 1984; Miller et al., 1984b). It has recently become possible to study  $\text{HCO}_3^-$  transport in isolation with the discovery that carbonyl sulphide ( $\text{COS}$ ), a close

structural analogue of  $\text{CO}_2$ , inhibited  $\text{CO}_2$  transport much more powerfully than  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  transport in *Synechococcus* UTEX625 (Miller et al., 1989). COS proved to be a substrate for the  $\text{CO}_2$  transport mechanism and was quantitatively hydrolysed to  $\text{H}_2\text{S}$  and  $\text{CO}_2$  in the light (Miller et al., 1989).  $\text{H}_2\text{S}$  has also been shown to selectively inhibit active  $\text{CO}_2$  transport in *Synechococcus* UTEX625 but, unlike COS did not appear to serve as a substrate for transport (Espie et al., 1989).  $\text{H}_2\text{S}$  had little effect on  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  transport, and when used extracellular  $\text{CO}_2$  concentrations rose above their equilibrium level. In the absence of  $\text{H}_2\text{S}$  extracellular  $\text{CO}_2$  concentrations are far below their equilibrium levels, suggesting  $\text{CO}_2$  transport not only serves as a primary means of  $\text{C}_i$  acquisition for photosynthesis, but also serves to recover  $\text{CO}_2$  lost from the cell, derived from the intracellular dehydration of  $\text{HCO}_3^-$  (Espie et al., 1989).

The adaptation process when cells are transferred from high to low  $\text{CO}_2$  conditions has been shown to depend on protein synthesis. In *Anabaena variabilis* and *Anacystis nidulans* the adaptation has been shown to be much slower in the dark, is inhibited by the protein synthesis inhibitor spectinomycin, the RNA synthesis inhibitor rifampicin and the 70S ribosome inhibitor chloramphenicol (Marcus et al., 1982; Omata and Ogawa, 1986).

Although no components of the  $\text{C}_i$  concentrating mechanism have as yet been identified, the studies on  $\text{C}_i$  transport and the effect

of inhibitors has enabled workers to elucidate the possible components and mechanism of  $C_i$  transport (see below).

### 1.5.3 Mechanisms of transport

From inhibitor studies the role of PSI in  $C_i$  transport has been firmly established. Ogawa and Ogren (1985) suggested that in *Anabaena variabilis*  $C_i$  transport could be driven with near equal efficiency with energy derived from PSI, or with energy transferred to PSI following absorption by PSII. Kaplan et al. (1987) has since shown that light is required not only for energization but for a time-dependent activation of the  $C_i$  transporting system in *A. nidulans*, similar to the induction of photosynthesis seen in dark-light transitions. It was concluded that ATP produced from PSI drove  $C_i$  transport, whilst PSII provided reducing equivalents needed for activation of transport.

The mechanism of induction of the  $C_i$  concentrating mechanism is unknown. In *A. variabilis* both Marcus et al. (1983) and Kaplan (1985) found that the initiation of adaptation from high to low  $CO_2$  was markedly affected by the  $O_2/CO_2$  concentration ratio, rather than  $O_2$  or  $CO_2$  concentration, suggesting that a metabolic product of RuBisCO might act as the signal for adaptation. This suggestion is supported by the fact that phosphoglycollate, the first product of the oxygenation reaction accumulates in cells following transfer from high to low  $CO_2$ .

Miller et al. (1984a) reported that induction of the  $C_i$



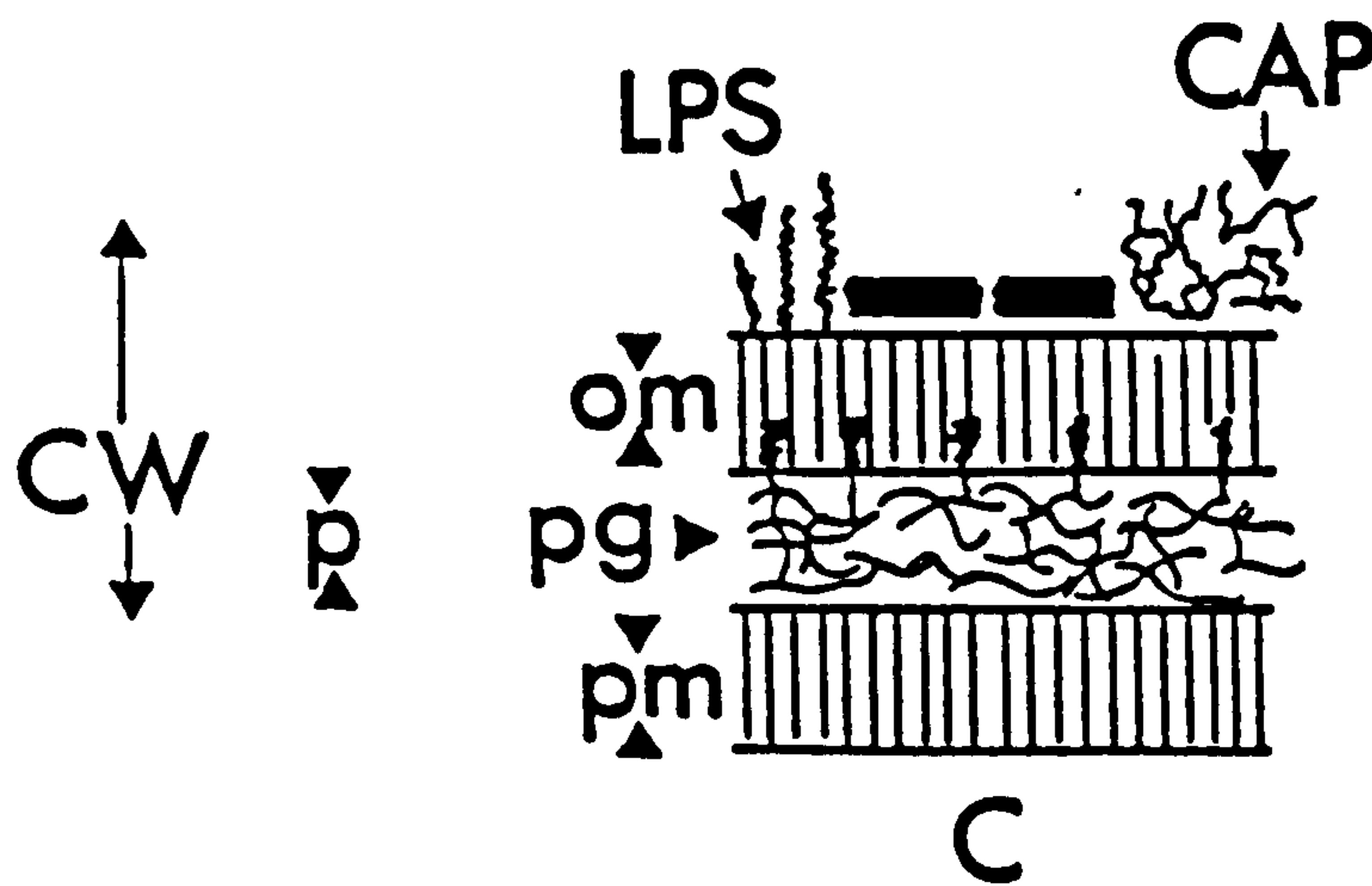
concentrating mechanism is a function of total DIC in the growth medium, suggesting that as in the transport of sulphate, in which a periplasmic protein has been implicated in the perception of sulphate levels in the medium (Green et al., 1989), a periplasmic protein senses external Ci levels.

As prokaryotes, containing one major internal compartment, the cytosol, separated from the external medium by the inner and outer cell membranes, it is assumed that the pumping mechanism is associated with this double membrane. The cell wall of cyanobacteria is of the Gram-negative type (Drews and Weckesser, 1982) (Fig. 1.6). As already mentioned many of the early workers assumed that  $\text{HCO}_3^-$  was the form of Ci transported. Kaplan et al. (1982) studied the response of the membrane potential to  $\text{HCO}_3^-$  supply with the aid of the lipophilic cation tetraphenyl phosphoniumbromide. They found that addition of  $\text{HCO}_3^-$  to  $\text{CO}_2$ -depleted cells resulted in a rapid hyperpolarization of the membrane, and concluded that a primary electrogenic pump was involved in the mechanism for  $\text{HCO}_3^-$  uptake, a view supported by Kaplan (1985) and Ogawa and Kaplan (1987). Badger and Andrews (1982) were the first to propose that the pump was able to directly use  $\text{CO}_2$  and  $\text{HCO}_3^-$  as substrates for a single complex transport mechanism. Volokita et al. (1984), assessing what was known about the pump, proposed a model for Ci transport in *Anabaena variabilis* (Fig. 1.7A) in which the pump was primarily envisaged as a  $\text{HCO}_3^-$  porter, active transport of  $\text{CO}_2$  involving the conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$  in the membrane by a "CA-like moiety", since ethoxycarbonyl amide inhibited  $\text{CO}_2$  transport



Figure 1.6 : Schematic representation of section through the cell envelope of a typical Gram negative bacterium

## Gram-negative



C - cytoplasm; PM - plasma membrane; PG - peptidoglycan; P - periplasm; OM - outer membrane; CW - cell wall; LPS - polysaccharide chains of lipopolysaccharide; CAP - capsule.

From Pugsley and Schwartz (1985)

preferentially over  $\text{HCO}_3^-$  uptake. A feature of this model is that it is  $\text{HCO}_3^-$  that arrives in the cytoplasm during both  $\text{HCO}_3^-$  and  $\text{CO}_2$  transport, since they found that intracellular pools of Ci as high as 15 mM were not saturating for photosynthesis. Badger et al. (1985) proposed a model for  $\text{HCO}_3^-$  accumulation in *Synechococcus* sp. Nageli, in which CA is necessary internally to allow a sufficiently fast rate of  $\text{CO}_2$  production to prevent a large accumulation of  $\text{HCO}_3^-$ , which was supported by *in vivo* and *in vitro* CA assays. These *in vivo* assays were the first to show the dehydration of  $\text{HCO}_3^-$  to  $\text{CO}_2$  was a catalyzed process in intact cyanobacteria.

As the presence of CA was firmly established in cyanobacteria, similar mechanisms to that proposed by Volokita et al. (1984), but incorporating CA, instead of a "CA-like moiety", as an essential element in the model, have also been proposed by Abe et al. (1987b), again in *A. variabilis*, and by Ogawa and Kaplan (1987) in *Synechococcus* PCC7942.

However, the observation that high and low affinity cells of *Synechococcus* PCC7942 (Badger and Gallacher, 1987; Price and Badger, 1989a,b) and *Synechococcus* UTEX625 (Miller and Canvin, 1987) differ most significantly in their abilities to transport  $\text{HCO}_3^-$ , with  $\text{CO}_2$  transport being constitutive, makes the model proposed by Volokita et al. (1984) unlikely, since as eloquently stated by Miller (1990) "it becomes unclear why high Ci grown cells should be able to actively transport  $\text{CO}_2$  with a  $\text{CO}_2$ -porter, whilst low Ci grown cells require a new CA-like porter to convert the  $\text{CO}_2$  to  $\text{HCO}_3^-$  so it can be passed onto a

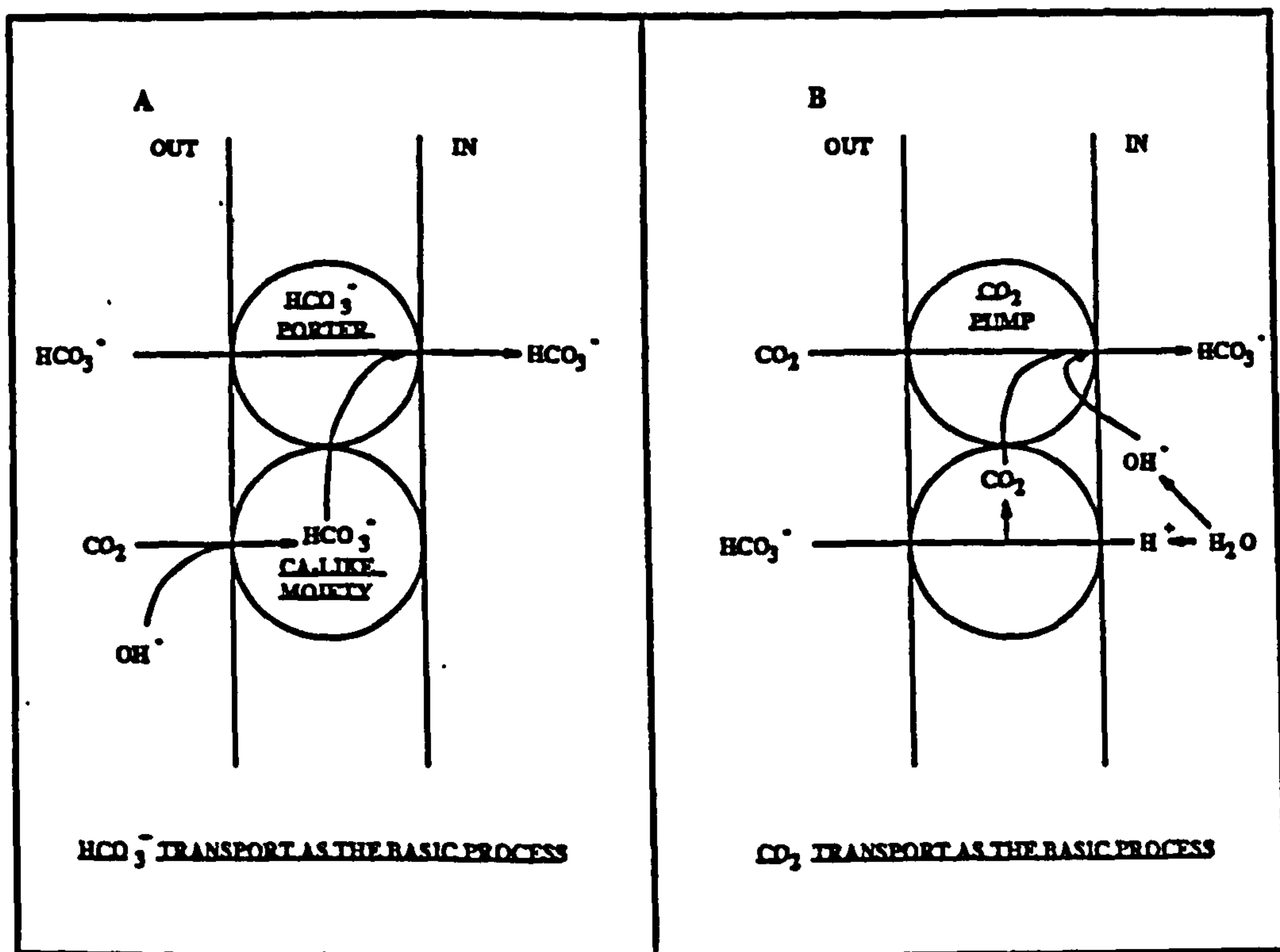


Figure 1.7. Two models that have been proposed for the transport of both CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>. Model (A) is based upon a diagram in Volokita *et al.* (1984) and model (B) upon a diagram in Price and Badger (1989a). (From Miller, 1990).

HCO<sub>3</sub><sup>-</sup> porter".

Price and Badger (1989a,b) have found, unlike Volokita et al. (1984), that the CA inhibitor ethoxycarbonyl amide (200 µM) inhibited both CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> uptake to a similar degree in low Ci cells of *Synechococcus* PCC7942. Using this observation, and the fact that it is CO<sub>2</sub> transport that is constitutive, Price and Badger (1989a) have proposed a model in which CO<sub>2</sub> transport is the basic process, delivering HCO<sub>3</sub><sup>-</sup> to the cell interior through the action of a CA-like step associated with the transport step, internal CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> equilibria maintained by internal CA (Fig. 1.7B). The presumed CA activity of these models is cryptic in terms of normal extracellular CA assays, as unlike green algae, no extracellular CA activity has been detected in cyanobacteria (Badger et al., 1985; Miller et al., 1988b).

The role of Na<sup>+</sup> in the Ci concentrating mechanism is unclear. It has been demonstrated that both *A. variabilis* (Kaplan et al., 1984) and *Synechococcus* UTEX625 (Miller et al., 1984b; Miller and Calvin, 1985) can be grown so that millimolar concentrations of Na<sup>+</sup> are required for HCO<sub>3</sub><sup>-</sup> transport, and that other monovalent cations cannot replace this dependence on Na<sup>+</sup>, Li<sup>+</sup> in fact being a competitive inhibitor with respect to Na<sup>+</sup> (Kaplan et al., 1984; Espie et al., 1988b). These requirements for Na<sup>+</sup> in *Synechococcus* UTEX625 can be overcome by high external Ci concentrations (Miller et al., 1984b; Espie et al., 1988b) or at low external Ci concentrations by the addition of CA (Miller et al., 1990), suggesting an intrinsic role for this cation in the



Ci concentrating system, and schematic models incorporating an  $\text{Na}^+$  gradient in bicarbonate uptake have been proposed (Kaplan et al., 1984; Reinhold et al., 1984; Kaplan, 1985). The uptake of Ci by *Thiobacillus neopolitanus*, apparently as  $\text{CO}_2$ , may involve a cation symport system, and  $\text{Na}^+$  at low concentrations stimulated  $\text{CO}_2$  transport in *Synechococcus* UTEX625 (Miller and Canvin, 1987; Espie et al., 1988b), though there was no evidence of concomitant  $\text{Na}^+$  uptake (Miller and Canvin, 1987). However both *Synechococcus* UTEX625 and *A. variabilis* can be grown to be  $\text{Na}^+$ -independent (Espie and Canvin, 1987; Kaplan et al., 1982). Miller (1989) thought it unlikely that a  $\text{Na}^+$  gradient is routinely involved in driving  $\text{HCO}_3^-$  transport, but that  $\text{Na}^+$  may serve as a counter-ion for  $\text{HCO}_3^-$  influx.

Evidence for separate transport systems for  $\text{CO}_2$  and  $\text{HCO}_3^-$  comes from results showing that the onset of rapid  $\text{HCO}_3^-$  transport, caused by the addition of  $\text{Na}^+$ , has little or no effect on the ongoing rate of  $\text{CO}_2$  transport at low  $\text{CO}_2$  concentrations (Espie et al., 1988a) and that the  $\text{CO}_2$  analogues carbonyl sulphide (COS) and  $\text{H}_2\text{S}$  selectively inhibit  $\text{CO}_2$  transport and leave  $\text{HCO}_3^-$  transport unaffected (Ogawa and Togasaki, 1988; Miller et al., 1989; Espie et al., 1989). COS was quantitatively hydrolysed to  $\text{CO}_2$  and  $\text{H}_2\text{S}$  in the light, which was explained in terms of a  $\text{CO}_2$  pump whose operation involves an obligatory hydration of  $\text{CO}_2$  (Miller et al., 1989), similar to the model proposed by Price and Badger (1989a) (Fig.1.7B). As well as the two models illustrated in Figure 1.7, a variety of other models of  $\text{CO}_2$  and

$\text{HCO}_3^-$  transport has been presented in the review by Miller (1990).

The recent work of Ogawa (1991) would seem to infer a close linkage between the transport of  $\text{HCO}_3^-$  and  $\text{CO}_2$ , since a mutation in the putative  $\text{Ci}$  transport gene *ictA* resulted in a mutant M9 unable to transport either of these  $\text{Ci}$  species.

The active transport of the  $\text{CO}_2$  molecule has only been confirmed in cyanobacteria and the green alga *Chlamydomonas reinhardtii* (Marcus et al, 1984; Sultemeyer et al., 1989), and is a process far different from the usual diffusive movements of  $\text{CO}_2$  into most photosynthetic cells (see Colman and Espie, 1985). The rates of photosynthetic  $\text{CO}_2$  fixation seen in cyanobacteria could only be supported by very high external  $\text{CO}_2$  concentrations if diffusive uptake of  $\text{CO}_2$  were occurring.

Espie et al (1991) found that the  $\text{CO}_2$  transporter in *Synechococcus* UTEX625 had a very high affinity (three times greater than the DIC saturated rate of photosynthetic  $\text{O}_2$  evolution under the same conditions) and was responsible not only for the initial transport of  $\text{CO}_2$  into the cell, but also for scavenging any  $\text{CO}_2$  that may leak from the cell during photosynthesis.

The location of the  $\text{Ci}$  pump is thought to be on the inner cell membrane. It has been reported in *Anabaena variabilis* (Marcus et al., 1982) that adaptation of high  $\text{CO}_2$ -grown cells to low  $\text{CO}_2$  conditions required light and protein synthesis. They observed

that high  $\text{CO}_2$  grown cells were more sensitive to lysozyme treatment and the composition of the cell membrane was different, being thicker in low  $\text{CO}_2$ -grown cells. Zenvirth and Kaplan (1984) showed that the rate of photosynthesis and the initial rates of  $\text{Ci}$  accumulation were very close in sphaeroplasts to those observed in intact cells. The steady-state intracellular  $\text{Ci}$  pool was however smaller in spheroplasts than intact cells, leading them to conclude that the cell wall does not play an essential role in  $\text{Ci}$  uptake, but may have a considerable impact on the diffusional dissipation of the intracellular  $\text{Ci}$  pool.

Omata and Ogawa (1985 and 1986) found a 42 kD protein in the cytoplasmic membrane of *A. nidulans* R2 thought to be associated with the  $\text{Ci}$  pumping mechanism. This polypeptide increased in parallel with the adaptation from high to low  $\text{CO}_2$  conditions (Omata and Ogawa, 1985) and spectinomycin inhibited both the  $\text{Ci}$  transporting capability of the cell and also the appearance of this protein. However, by the use of a mutant of *A. nidulans* R2 incapable of synthesizing this protein, but capable of  $\text{HCO}_3^-$  and  $\text{CO}_2$  transport, Schwarz et al. (1988) have convincingly demonstrated that this protein has no obvious role in  $\text{Ci}$  uptake, and so as of yet, no constituents of the  $\text{Ci}$  pump have been identified.



#### 1.5.4 The role of carboxysomes

If the active accumulation of  $C_i$  is to be effective, raising the concentration of  $CO_2$  to high levels around the active sites of RuBisCO, the leakage of  $CO_2$  from the cells must be prevented or decreased. Carbon dioxide has a high lipid solubility and it is relatively permeable to biological membranes (Gutknecht et al., 1977), and hence the problem facing cyanobacteria is how to maintain a high internal  $CO_2$  concentration without the need for a massive energy expenditure to overcome the rapid leakage of  $CO_2$  (see Raven and Lucas, 1985). Volokita et al. (1984) proposed that the majority of the internal  $C_i$  pool was in the form of  $HCO_3^-$ , which is much more membrane impermeable than  $CO_2$  (Gutknecht et al., 1977), since pool sizes as large as 15 mM were not saturating for photosynthesis, and RuBisCO is known to have a  $K_m$   $CO_2$  of 0.2-0.3 mM. Under steady state photosynthesis, Badger and Andrews (1982) found by monitoring the  $CO_2$  in solution that upon illumination there was a sharp decline in  $CO_2$  in the medium, followed by an apparent rise in  $CO_2$  level, and then a gradual decline to zero  $CO_2$  and zero  $C_i$ . This evolution of  $CO_2$  occurred simultaneously to net  $O_2$  evolution and  $CO_2$  fixation. When they switched off the light there was a rapid evolution of  $CO_2$  which was eliminated by CA, suggesting the  $CO_2$  efflux is coming from a pool of concentrated  $CO_2$  within the cell.

The maintenance of a high intracellular  $CO_2$  concentration is due to the low conductance of the cell envelope to the passive diffusion of  $CO_2$ . Conductance values of  $10^{-5}$  cm sec<sup>-1</sup>, several

orders of magnitude lower than previously reported for any cell or bilayer lipid membrane, have been reported in cyanobacteria (Badger et al., 1985). This low passive permeability to  $\text{CO}_2$  explains how high concentrations of  $\text{CO}_2$  can be maintained around RuBisCO without a back leakage so high as to place impossible energetic demands on the cell (Raven and Lucas, 1985). At first it was thought that this barrier to  $\text{CO}_2$  leakage resided in the plasmalemma. However as Badger et al. (1985) also mentioned, if the diffusion of  $\text{O}_2$  were to be restricted to the same degree, it would be likely that toxic concentrations of  $\text{O}_2$  would develop in the cells during photosynthesis. In addition, Walsby (1985), using a technique based on the collapse of gas vacuoles under pressure, has estimated vegetative cells of *Anabeana* to have a permeability to  $\text{N}_2$  of  $10^{-3} \text{ cm s}^{-1}$ , which would make  $\text{N}_2$  100 fold more permeable than  $\text{CO}_2$ , which is unlikely.

A hypothesis has been put forward that obviates the dilemma regarding the selective permeability of gases in cyanobacteria. This hypothesis (Reinhold et al., 1987) proportions a major functional role to the carboxysomes. Carboxysomes are subcellular inclusion bodies, 100-200 nanometers (nm) in diameter, with a 3-4 nm thick shell surrounding them (Codd and Marsden, 1984). The structure of carboxysomes from *Thiobacillus neopolitanus* has been determined (Holthuisen et al., 1986), and it appears that the protein layer surrounding them contains no lipid. Most of the functional RuBisCO of cyanobacteria grown under conditions of limiting  $\text{CO}_2$  concentration, is contained in these polyhedral bodies (Codd and Marsden, 1984). In addition to

RuBisCO, which can account for up to 50% of total protein, SDS-PAGE has shown them to contain a very limited number of polypeptides, none of which are Calvin cycle enzymes (Codd, 1988).

The model by Reinhold et al. (1987) postulates

- 1) It is  $\text{HCO}_3^-$  that arrives in the cytoplasm, irrespective of the Ci species transported.
- 2) CA is found only in the carboxysomes, not the cytoplasm.
- 3) In the carboxysome resistance to  $\text{HCO}_3^-$  and RUBP uptake, and to 3-PGA and  $\text{OH}^-$  efflux are reasonably low ( $P[\text{HCO}_3^-] = 10^{-4} \text{ cm s}^{-1}$ ).
- 4) Resistance to the efflux of  $\text{CO}_2$  generated from CA catalyzed  $\text{HCO}_3^-$  dehydration within the carboxysome is high ( $P[\text{CO}_2] = 10^{-5} \text{ cm s}^{-1}$ ).

According to the model  $\text{HCO}_3^-$  builds up to high levels in the cytoplasm, and diffuses into the carboxysome, where it is dehydrated to  $\text{CO}_2$  by carbonic anhydrase and subsequently utilized by RuBisCO. The model has a considerable amount of evidence to support it.

Codd and Stewart (1976) found that carboxysomes isolated by differential centrifugation were capable of RUBP-dependent  $\text{CO}_2$  fixation and so appear to be permeable to RUBP.

Phosphoribulokinase, which produces RUBP by phosphorylating ribulose-5-phosphate is not found in the carboxysome, but in the cytoplasm (Hawthornthwaite et al., 1985), consistent with the cytoplasmic location and operation of most of the Calvin cycle enzymes this model postulates.

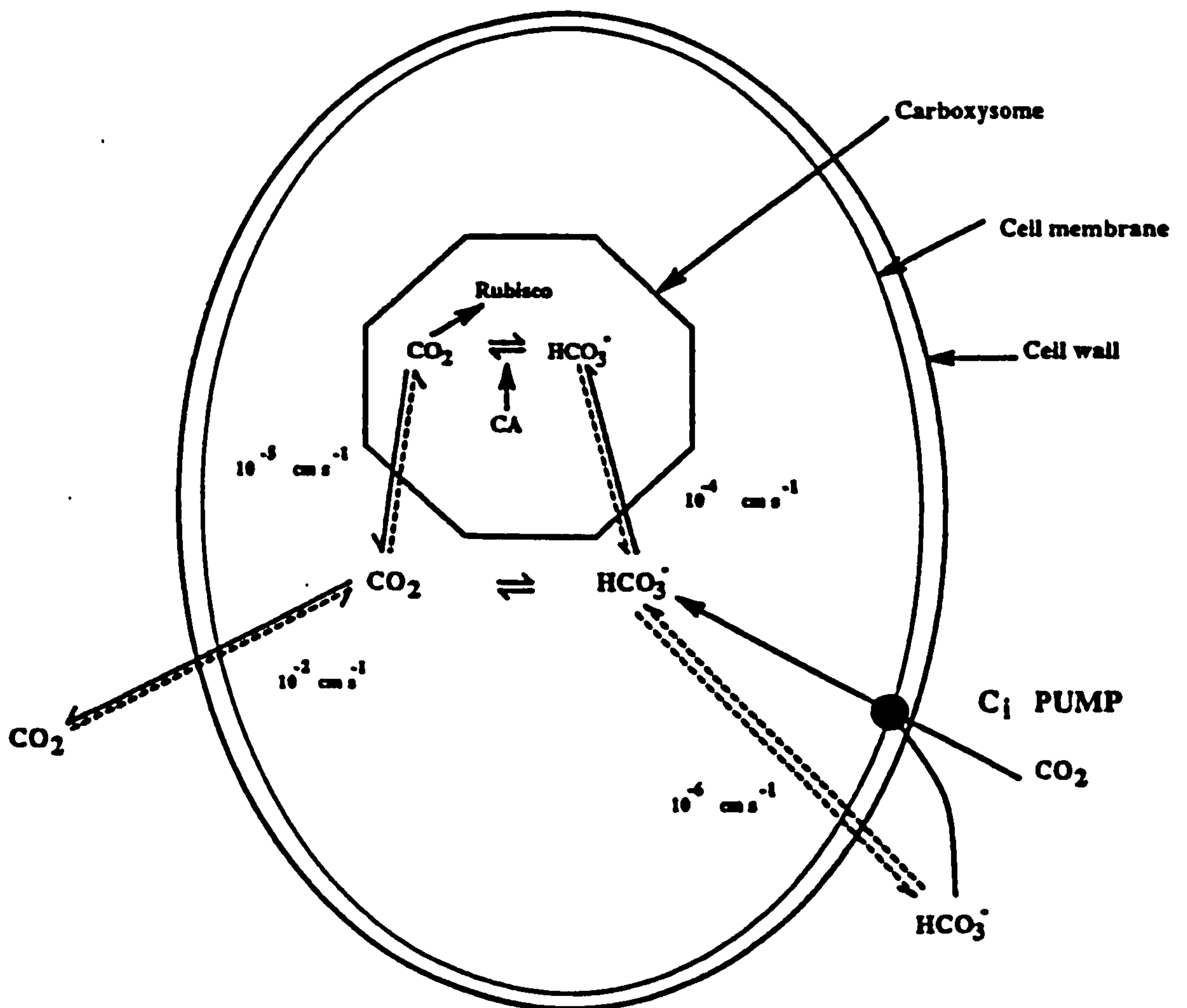


The actual location of CA is more uncertain. Yagawa et al. (1984) found some strains of *Anabaena* had only soluble CA whereas other strains had soluble and insoluble CA, and Lanaras et al. (1985) suggested that the CA in *Chlorogloeopsis fritschii* was not specifically associated with the carboxysome or thylakoids. However, Badger and Price (1989) found in *Synechococcus* PCC7942 that some, but not all, of the CA activity pelleted with the RuBisCO activity on sucrose gradients. Price and Badger (1989c) expressed the gene for the human carbonic anhydrase II (HCA II) protein in *Synechococcus* PCC7942 by transformation with the bidirectional expression vector pCA. This expression was driven by the bacterial *Tac* promoter and was regulated by the *lacIQ* repressor protein, expressed from the same plasmid. Expression levels reached 0.3% of total cell protein and the protein appeared to be soluble in nature, located in the cytosol. They have suggested that expression of CA in both high and low  $C_i$  grown cells leads to the creation of a high  $C_i$  requiring phenotype since  $CO_2$  is partitioned away from RuBisCO by the carboxysome coat - experimentally the  $K_m$  photosynthesis increased, there was a loss in the ability to accumulate  $C_i$  internally and there was a decrease in the lag between initial  $C_i$  accumulation following illumination and the efflux of  $C_i$  from the cells. The effects of expressed CA could largely be overcome by the addition of ethoxycarbamide, adding further credence to the suggestion that the  $CO_2$  concentrating mechanism in *Synechococcus* PCC7942 is largely dependent on the

absence of CA activity from the cytosol and its specific localisation in the carboxysome. They have produced a model (see Fig. 1.8) in which the carboxysome plays a central role as both the site of  $\text{CO}_2$  generation from  $\text{HCO}_3^-$  and of resistance to  $\text{CO}_2$  efflux from the cell. The same authors (Price and Badger, 1989d) carried out analysis of 24 high  $\text{CO}_2$ -requiring mutants of *Synechococcus* PCC7942, and classified them into two types depending on their phenotype. The features of type I mutants were most consistent with a scenario where CA had been mistargetted to the cytosol, whilst those of type II were most consistent with a scenario where CA was missing from the cell. However CA activity had been found in crude extracts, and so type II mutants were thought to have a lesion in their capacity for  $\text{H}^+$  import during photosynthesis.

There are also however arguments against this hypothesis. The confinement of CA to the carboxysome has not been proven. In fact in more cases than not it has been found to be soluble (Yagawa et al., 1984; Lanaras et al., 1985) and both Badger and Price (1989) and Kaplan (1990) found separate soluble and pelletable CA activity. Miller et al. (1990) using COS as a specific  $\text{CO}_2$  transport inhibitor found that on addition of  $^{13}\text{C}^{18}\text{O}$ -labelled  $\text{HCO}_3^-$  for 5 sec followed by COS there was, within 3 sec, a large efflux of  $^{13}\text{CO}_2$  suggesting an intracellular concentration of at least 0.6 mM, and in cells undergoing steady-state photosynthesis, the rapid  $\text{CO}_2$  efflux suggested an intracellular  $\text{CO}_2$  pool of at least 1 mM. As

Figure 1.8 : A model of cyanobacterial photosynthesis, incorporating the carboxysome as central location for  $\text{CO}_2$  elevation within the cell. The major direction of  $\text{CO}_2$  and  $\text{HCO}_3^-$  fluxes is indicated by bold arrows, and the fluxes are given the conductance values shown. (From Price and Badger, 1989c).





carboxysomes occupy no more than 5% of the cell volume (Price and Badger, 1989a), Miller et al. (1990) argued that if  $\text{CO}_2$  was restricted here, very high concentrations would exist, which would probably require active  $\text{HCO}_3^-$  transport, which considering the very limited number of proteins they contain was unlikely. In addition, most of the  $^{13}\text{CO}_2$  leaked from the cell was not labelled with  $^{18}\text{O}$ , indicative of a rapid loss of  $^{18}\text{O}$  which must have been catalyzed by CA activity. Thus there is the possibility that not only is the intracellular  $\text{CO}_2$  concentration high, but also that it is in rapid equilibrium with total intracellular  $\text{HCO}_3^-$ , mediated by CA (Miller et al., 1990).

At present, results as to the location and functioning of the Ci concentrating mechanism are conflicting, and it is hoped that genetic and molecular biological studies will help solve these issues. Present evidence tends to favour the central role of the carboxysome. As well as the evidence of Price and Badger (1989c,d), a number of other workers have implicated carboxysomes in the Ci concentrating mechanism. A mutant of *Synechocystis* PCC6803, which had its RuBisCO gene replaced with the RuBisCO gene from *Rhodospirillum rubrum* was found to require high  $\text{CO}_2$  levels for growth, was extremely sensitive to the  $\text{CO}_2/\text{O}_2$  ratio supplied during growth, and was found to lack all microscopically observable carboxysomes (Pierce et al., 1989). A number of high  $\text{CO}_2$ -requiring mutants of *Synechococcus* PCC7942 have been isolated. Two, mutant E<sub>1</sub> (Marcus et al., 1986) and O<sub>221</sub> (Schwarz et al., 1988) are capable of accumulating Ci

internally almost as efficiently as the WT, yet they exhibit a low photosynthetic affinity for extracellular Ci and demand high CO<sub>2</sub> for growth as they are defective in utilising the internal Ci pool. Both mutants appear to possess defective carboxysomes (Friedberg et al., 1989; Kaplan, 1990), and when transformed with PE12, a plasmid containing 10 kb of WT DNA, grew at air levels of CO<sub>2</sub>, the physiological and biochemical characteristics, including carboxysomes, resembling those of WT cells (Kaplan, 1990). A 3.5kb ClaI fragment of PE12 transformed mutant O<sub>221</sub>, but not mutant E<sub>1</sub>. The ClaI fragment has been shown by sequence analysis to contain part of *rbcl*, the structural gene encoding the large subunit of RuBisCO, and its 5'-flanking region, the data suggesting a possibility that this region of DNA might be involved in the functional organisation of the carboxysome (Kaplan, 1990).

## 1.6 The inorganic carbon concentrating mechanism in other organisms

### 1.6.1 Green algae

The unicellular green alga *Chlamydomonas reinhardtii* was the first aquatic organism in which the CO<sub>2</sub> concentrating mechanism was identified (Berry et al., 1976). Similar results were also seen in *Scenedesmus obliquus* (Findenegg, 1976) and *Chlorella vulgaris* (Hogetsu and Miyachi, 1977).

This increased affinity for external Ci for cells grown with air levels of CO<sub>2</sub>, resulting in increased photosynthesis and reduced photorespiration was shown to be associated with the ability to

concentrate  $C_i$  internally (Badger et al., 1980; Beardall and Raven, 1981; Moroney and Tolbert, 1985). As in cyanobacteria, this  $C_i$  concentrating mechanism was inducible, induction occurring at air but not elevated (1-5%) levels of  $CO_2$ . (Berry et al., 1976; Badger et al., 1980). Biochemical and physiological studies have implicated the involvement of at least two components, an energy dependent, saturable  $C_i$  transport process (Spalding et al., 1983) and the enzyme CA (Badger et al., 1980; Spalding et al., 1983).

Experiments with different algal species have indicated that CA can be located in at least three different cell compartments. CA activity has been found associated with the chloroplast (Hogetsu and Miyachi, 1979), the cytoplasm (Findenegg, 1979) and the extracellular space (Kimpel et al., 1983).

Many studies have shown that CA is an important component of the  $C_i$  concentrating system in green alga (see Aiazawa and Miyachi, 1986). In *C. reinhardtii* there is a correlation between induction of the  $C_i$  concentrating system and increased extracellular levels of CA (Kimpel et al., 1983; Coleman et al., 1984). Using photosynthetic mutants of *C. reinhardtii* and manipulations in the growth regime of WT cells, Spalding and Ogren (1982) coordinated the regulation of CA activity and  $C_i$  transport. Spalding et al. (1983a) reported that high  $CO_2$ -grown cells of *C. reinhardtii* have little if any CA activity associated with the periplasmic space, whilst air grown cells have much CA activity in the periplasmic space and some intracellular CA activity. Moroney et al. (1987) found that both air and high



CO<sub>2</sub>-grown *C. reinhardtii* have a physiologically important internal CA, whilst only air-grown cells possess the major CA located in the periplasmic space, and concluded that the internal CA is under different regulatory control from the periplasmic CA. Husic et al. (1989) found that the susceptibility of the intracellular CA is more than three orders of magnitude less than the periplasmic CA to CA inhibitors such as EZ and AZ, indicating that the intracellular CA is distinct from the periplasmic form of the enzyme. Southern blot analysis of the gene coding for CA in *C. reinhardtii* indicated that only a single copy of the gene is present (Bailly and Coleman, 1988). The 2.5 kb DNA fragment encoding the CA gene hybridised to a 1.4 kb mRNA transcript. It was found that maximum levels of this transcript occurred after 3 hours exposure of high CO<sub>2</sub>-grown cells to air, whilst transfer of air-grown cells to high CO<sub>2</sub> resulted in elimination of the transcript after 60 minutes (Bailly and Coleman, 1988).

It has long been known that active HCO<sub>3</sub><sup>-</sup> transport occurs in *C. reinhardtii* (Badger et al., 1980; Williams and Turpin, 1987). It was thought by many workers that CO<sub>2</sub> uptake involved diffusion across the plasmalemma with the CA mediated conversion of CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup> maintaining the diffusion gradient (Moroney and Tolbert, 1985; Patel and Merrett, 1986). It has been shown that intact chloroplasts isolated from a wall-less mutant of *C. reinhardtii* accumulate Ci, provided the cells had been adapted to low CO<sub>2</sub>-growth conditions (Moroney et al., 1987). They presented a model in which active Ci transport, in the form of HCO<sub>3</sub><sup>-</sup>

occurred only at the chloroplast envelope. However, the fact that the uptake of  $\text{CO}_2$  from the external medium is faster than that of  $\text{HCO}_3^-$  led some workers to conclude that both species of Ci were actively transported (Williams and Turpin, 1987). This has now been proven in *C. reinhardtii* by the use of MIMS which has shown that both  $\text{HCO}_3^-$  and  $\text{CO}_2$  are actively transported, with  $\text{CO}_2$  being preferentially used (Sultermeyer et al., 1989). A number of mutants of *C. reinhardtii* defective in Ci transport have been isolated and their use in analysing the Ci concentrating mechanism is reviewed in section 6.1.2. Four major proteins have been identified which were preferentially synthesized under low  $\text{CO}_2$  conditions in *C. reinhardtii* (Bailly and Coleman, 1988; Manuel and Moroney, 1988). One was identified as the periplasmic CA, and none were synthesized by the mutant *pmp-1* which cannot grow at low  $\text{CO}_2$  concentrations, suggesting they may be involved in the Ci concentrating mechanism (Manuel and Moroney, 1988).

#### 1.6.2 Other species

*Thiobacillus neapolitanus* is capable of the active transport of Ci, resulting in an accumulation of Ci internally 1000-1500 fold greater than the external environment in  $\text{CO}_2$  limited cells and 500-800 fold in thiosulphate limited cells (Holthuizen et al., 1986). It has long been known that the leaves of aquatic angiosperms such as *Potamogeton*, *Elodea* and *Valisneria* are able to assimilate  $\text{HCO}_3^-$  during photosynthesis (see Steemann-Nielsen, 1960), and a number of models for the photosynthetic utilization

of  $\text{HCO}_3^-$  have been proposed (see Badger, 1987). Lucas (1975) showed that in *Chara corallina* transport of bicarbonate occurred because in a closed system  $\text{O}_2$  evolution exceeded the rate of  $\text{CO}_2$  supply. It has also been shown in some aquatic macrophytes there appears to be a Ci accumulation system working in conjunction with CA activity (Salvucci and Bowes, 1983), whilst more recently it has been proposed, from evidence on the influence of DIC and  $\text{O}_2$  concentrations on photosynthesis that *Chara corallina* cells are capable of accumulating  $\text{CO}_2$  internally by means of a process apparently independent of the plasmalemma  $\text{HCO}_3^-$  transport system (Brechignac and Lucas, 1987). In the marine red macroalga *Chondrus crispus* it has been shown that over 90% of photosynthesis is supported by  $\text{HCO}_3^-$  uptake (Brechignac et al., 1986), and that this Ci uptake involves both membrane-associated and internal CA (Smith and Bidwell, 1987). A more in-depth account of  $\text{HCO}_3^-$  utilization can be found in the review by Badger (1987)

### 1.7 Ecological significance of the Ci concentrating mechanism

As mentioned in section 1.3 the supply of  $\text{CO}_2$  to aquatic organisms can vary depending on the physiochemical nature of the aquatic environment (Round, 1981). In freshwater environments the total DIC,  $\text{CO}_2$ ,  $\text{HCO}_3^-$  and pH can all vary dramatically depending on the biological activity of the photosynthetic organisms that inhabit them (Talling, 1985). In water bodies of low photosynthetic activity neither carbon depletion nor biologically induced pH changes are likely to occur (Badger,



1987). However in productive water bodies, photosynthetic activity can exceed the supply of  $\text{CO}_2$  from the atmosphere, which will lead to a depletion in the amount of DIC and an increase in the pH of the water, often to values above 9 (Talling, 1985) where the concentration of free  $\text{CO}_2$  will be at values well below equilibrium (see Fig. 1.5). Under these conditions the  $\text{Ci}$  concentrating mechanism will be very important as an adaptive mechanism, since the ability to photosynthesize at low  $\text{Ci}$  concentrations and to access the  $\text{HCO}_3^-$  pool will be vital for sustained growth. In natural situations there appears to be a gradation of abilities of organisms to grow under increasing levels of  $\text{CO}_2$  depletion and alkalinity, with cyanobacteria the most successful under conditions of maximum  $\text{Ci}$  depletion (Talling, 1985), which may reflect their abilities to concentrate  $\text{Ci}$  internally to higher levels than green alga (see Badger et al., 1980; Kaplan et al., 1980).

It has already been briefly mentioned that cyanobacteria have intermediate  $K_{0.5}\text{DIC}$  when grown in conditions that are intermediate between high and low  $\text{CO}_2$  growth regimes (see Turpin et al., 1985; Mayo et al., 1986). These intermediate DIC concentrations, between 40 and 1500  $\mu\text{M}$ , are often experienced in aquatic ecosystems, where cyanobacteria are expected to have intermediate  $K_{0.5}\text{DIC}$  values similar to the actual DIC concentration in the water (Turpin et al., 1985; Mayo et al., 1986). Such a situation would allow the cells to sense and respond to changes in the external DIC concentration by altering the kinetics of their  $\text{Ci}$  uptake mechanism (Turpin et al., 1985).

In the marine environment fluctuations in the concentration of the various  $C_i$  species and pH are not as significant as in freshwater environments, because seawater has a high and constant  $HCO_3^-$  concentration of around 1.8 mM, a pH of 8.2 and a  $CO_2$  concentration in equilibrium with air (14  $\mu$ M at 15°C) (Round, 1981). Under these conditions the potential  $CO_2$  supply rate for photosynthesis will be greater than that found in freshwater situations, and the  $C_i$  concentrating mechanism less important in utilising limited  $C_i$  supplies (Badger, 1987).

This thesis examines the responses of selected unicellular cyanobacteria to changes in their carbon regime at several levels. Initially the ability of several unicellular cyanobacteria, grown in batch culture under different carbon regimes, to transport  $C_i$  from the external environment into the internal  $C_i$  pool was characterised.

The  $C_i$  uptake mechanism was then studied under the more defined conditions of continuous culture in *Synechococcus* PCC7942, with particular attention also being focused on the role of the 42 kD cytoplasmic membrane polypeptide which was at one time thought to be involved in the  $C_i$  concentrating mechanism (see Omata and Ogawa, 1986). The possible role of protein phosphorylation (*in vivo* and *in vitro*) in the adaptive transformation was also examined, with particular emphasis given to elucidating the possible link between the appearance of phosphopolypeptides and the decline in the  $C_i$  uptake rate in low  $CO_2$ -grown cells of *Synechocystis* PCC6803 following addition of glucose or

bicarbonate. Finally a molecular genetic approach to studying the process was examined by the use of specially constructed *LacZ* promoter probes.



## Chapter 2

### Materials and Methods

The methods described here are those that were used routinely. Techniques specific to particular experiments and derivations of the following methods are described in the appropriate results chapter.

## 2.1 Organisms

All cyanobacterial strains used in this thesis were obtained from the culture collection maintained in Professor N.G.Carr's laboratory at Warwick University. A complete list of cyanobacteria, together with designation from the Pasteur Culture Collection (PCC) or that of the Woods Hole Oceanographic Institute (WH) is given in Table 2.1, along with the *Escherichia coli* strains used.

## 2.2 Plasmids

Plasmids used and constructed in this study are described in Table 2.2.

## 2.3 Reagents

All media, unless otherwise indicated, was made using BDH AnalR grade chemicals. Bacto-tryptone and Bacto-agar were obtained from Difco and yeast extract, nutrient agar and nutrient broth from Oxoid. Acrylamide and caesium chloride were obtained from Fisons plc, and N,N'-methylene bis-acrylamide from Eastman Kodak Co. Glycine, electrophoretic grade TEMED (N,N,N',N'-tetramethylethylene diamine), 2-mercaptoethanol,

sodium dodecyl sulphate (SDS), SDS-PAGE molecular weight markers and protein assay concentrate were from BioRad Laboratories. Silver nitrate was obtained from Johnson Matthey Chemicals Ltd. Calcium chloride dihydrate Grade I, Tris base, lysozyme, snake venom phosphodiesterase, adenosine triphosphate (ATP), protease K, bovine alkaline phosphatase, DNase I, RNase A, phenylmethylsulfonylfluoride (PMSF),  $\epsilon$ -amino-n-caproic acid, iodoacetamide, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 3-O-methyl-D-glucopyranose (OMG), 2-deoxy-D-glucose (DOG), acetazolamide, 4-methyl umbelliferyl- $\beta$ -D-galactoside (MUG), methyl umbelliferyl phosphate (MUP) and the buffers N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2-[N-morpholino]ethanesulfonic acid (MES), N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), N-tris[hydroxymethyl]methyl-3-propanesulfonic acid (TAPS), 2-[N-cyclohexylamino]ethanesulfonic acid (CHES) were from Sigma Chemical Company. Restriction enzymes and all radioisotopes except  $^{32}\text{P}$  gamma-labelled ATP (ICN Ltd.) were from Amersham International. Glass microfibre filters were from Whatman, silicone oils from Wacker-Chemie and scintillation fluid from Beckman.

#### 2.4 Growth media

Cyanobacterial strains were maintained under photo-autotrophic growth conditions. Freshwater organisms were grown in BG-11, whilst marine organisms were grown in ASN-III, a synthetic seawater media (see Rippka et al., 1979) and are listed in Table



2.3. All media was made using analytical grade chemicals and purified water from a double ion-exchange unit with carbon filter (Elgastat). Sterilization was achieved by autoclaving at 15 psi for 15 minutes, unless noted otherwise. Volatile and labile components were filter sterilized through Millipore GS disposable filters.

## 2.5 Growth and maintenance of cyanobacteria

All of the organisms originally designated from the PCC were grown at 30°C in an illuminated orbital shaker (Vindon Scientific Ltd) at a light intensity of 35-40  $\mu\text{E m}^{-2}\text{s}^{-1}$ , measured using a Macam 3000 photometer/radiometer (Macam photometrics Ltd), whilst those organisms from WH were grown in ASN-III at 25°C in stationary culture at a light intensity of 10-15  $\mu\text{E m}^{-2}\text{s}^{-1}$ . High  $\text{CO}_2$  conditions were obtained by the addition of 10 mM  $\text{NaHCO}_3$  and aeration with 5%  $\text{CO}_2$  in air (v/v). Low  $\text{CO}_2$  cultures were grown in media with no mineral carbon, atmospheric  $\text{CO}_2$  being the sole carbon source. Heterotrophic growth was achieved by the addition of 10 mM glucose (filter sterilized) to BG-11 and then  $10^{-5}\text{M}$  DCMU, followed by incubation as for low  $\text{CO}_2$  cultures.

For solid media, BG-11 was supplemented with 1.5% (w/v) bacto agar. Double strength agar and growth medium were autoclaved separately and mixed at approximately 50°C. Plates were incubated at 30°C (controlled temperature room), approximately 10 cm away from two warm white strip lights (Osram 65/80 W). Cultures originally from the PCC were maintained on 1.5% (w/v)

agar slopes of BG-11 in the laboratory, avoiding direct sunlight, whilst those from WH were maintained in stationary liquid culture, at a light intensity of  $4-5 \text{ uE m}^{-2} \text{ s}^{-1}$ . Absence of contamination was checked by plating stock cultures onto nutrient agar or the appropriate growth medium, supplemented with 10 mM glucose and 0.2% (w/v) yeast extract, incubating in the dark at either 25 or 30°C, depending on the organism. Absence of bacterial or fungal growth after 7 days was taken to indicate an axenic culture.

## 2.6 Growth and maintenance of *Escherichia coli*

*E. coli* was grown in Oxoid nutrient broth (13g/litre) at 37°C in a Gallenkamp shaking incubator at 200 rpm. Solid media was made using Oxoid nutrient agar (15g/litre). For high efficiency of transformation SOB medium was used, containing

g l<sup>-1</sup>

Bactotryptone.....	20.0
yeast extract.....	5.0
NaCl.....	0.5844
KCl.....	0.186

To this was added 10 ml of a filter sterilized solution consisting of 1M MgSO<sub>4</sub>, 1M MgCl<sub>2</sub>.

Cultures were stored on nutrient agar for 1-2 months at 4°C, or at -20°C in 15% (w/v) glycerol for long term storage.

Table 2.1      Organismsa) Cyanobacteria

<u>Strain</u>	<u>Source</u>	<u>Alternative name</u>
<i>Synechococcus</i> R2	PCC7942	<i>Anacystis nidulans</i>
<i>Synechococcus</i> R2-SPc	PCC7942	(small plasmid cured)
<i>Synechococcus</i> sp.	PCC6301	<i>Anacystis nidulans</i> , <i>Synechococcus leopoliensis</i> (UTEX 625)
<i>Synechocystis</i> sp.	PCC6803	<i>Aphanocapsa</i> sp.
<i>Synechocystis</i> sp.	PCC6714	<i>Aphanocapsa</i> sp.
<i>Synechocystis</i> sp.	PCC6308	<i>Gloeocapsa alpicola</i>
<i>Synechococcus</i> sp.	PCC7002	<i>Agmenellum quadruplicatum</i> PR-6
<i>Synechococcus</i> sp.	PCC73109	<i>Agmenellum quadruplicatum</i> BG-1
<i>Synechococcus</i> DC2	WH7803	
<i>Synechococcus</i> sp.	WH8018	
<i>Synechococcus</i> sp.	WH8110	

b) *Escherichia coli*

<u>Strain</u>	<u>Source</u>
MC1061	Casabadan and Cohen (1980)



Table 2.2    Plasmids1) Cyanobacterial

<u>Plasmid</u>	<u>Characteristics</u>	<u>Source/Reference</u>
a) <u>Shuttle vectors</u>		
pUC105	Ap <sup>r</sup> , Cm <sup>r</sup>	Kuhlemeier et al., 1981
PUC303	Sm <sup>r</sup> , Cm <sup>r</sup>	Kuhlemeier et al., 1983

b) Promoter probes

pLACPB1	Ap <sup>r</sup> , Cm <sup>r</sup> , promoterless <i>lacZ</i>	D.J.Scanlan
PLACPB2	Ap <sup>r</sup> , Cm <sup>r</sup> , promoterless <i>lacZ</i> with transcription terminators	This study

c) Libraries

pLACPB1 ::	<i>Synechococcus</i> R2 chromosomal DNA	This study
pLACPB2 ::	<i>Synechococcus</i> R2 chromosomal DNA	This study

2) *Escherichia coli*

pBR322	Ap <sup>r</sup> , Tc <sup>r</sup>	Balbas et al., 1986
pDAH274	Ap <sup>r</sup> , Km <sup>r</sup> , promoterless <i>lacZ</i> , P1 <i>inc</i>	D.Hodgson

Table 2.3    Growth mediaBG-11

	<u>g l<sup>-1</sup></u>
NaNO <sub>3</sub>	1.50
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.075
K <sub>2</sub> HPO <sub>4</sub>	0.04
Na <sub>2</sub> CO <sub>3</sub>	0.02
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.036
EDTA	0.001
citric acid	0.006
FeNH <sub>4</sub> .citrate	0.006
microelements A5	1 ml l <sup>-1</sup>

Microelements A5    g l<sup>-1</sup>

H <sub>3</sub> BO <sub>3</sub>	2.86
MnCl <sub>2</sub> ·4H <sub>2</sub> O	1.81
NaMoO <sub>4</sub> ·2H <sub>2</sub> O	0.39
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.222
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.079
Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	0.049

ASN-III

	<u>g l<sup>-1</sup></u>
NaNO <sub>3</sub>	0.75
MgSO <sub>4</sub> ·7H <sub>2</sub> O	3.50
K <sub>2</sub> HPO <sub>4</sub>	0.02
Na <sub>2</sub> CO <sub>3</sub>	0.02
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.50
EDTA	0.0005
citric acid	0.003
FeNH <sub>4</sub> .citrate	0.003
NaCl	25.0
KCl	0.50
MgCl <sub>2</sub> ·6H <sub>2</sub> O	2.0
microelements A5	1 ml l <sup>-1</sup>

## 2.7 Measurement of growth by dry weight estimation

A known volume of cells was centrifuged at 10,000 rpm for 10 min, the pellet resuspended, and aliquots of the suspension vacuum filtered onto pre-weighed Whatman GF/F or Nucleopore polycarbonate filters (0.2  $\mu$ M). These were then dried to constant weight at 110°C. The extinction at OD<sub>750</sub> of various dilutions of the cell suspension were measured as well (Ultraspec II), and an OD<sub>750</sub> to dry weight relationship established.

## 2.8 Autotrophic continuous-flow culture

Figure 2.1 illustrates schematically the basic features of the continuous-flow culture system used in this study to provide the controlled autotrophic growth of *Synechococcus* PCC7942 under CO<sub>2</sub>-limited conditions, whilst Figure 2.2 shows the actual system in operation.

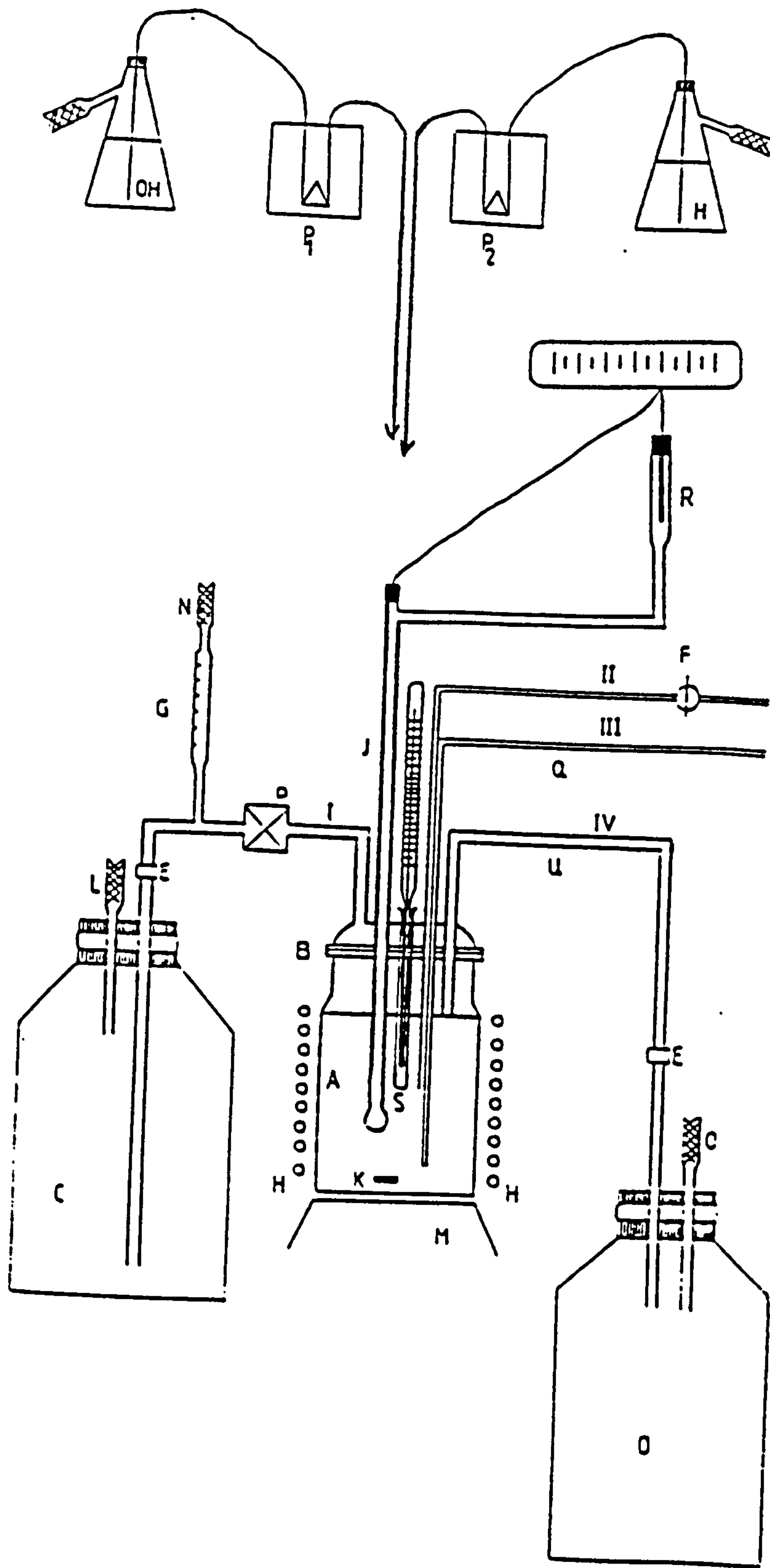
The culture vessel (A) was a 1.25 l Pyrex glass vessel (Quickfit, England) with a working culture volume of 1.0 l. The top of the culture vessel was fitted with a glass lid (B), secured rigidly in position by a metal ring, which was placed over the flanged lip of the lid and culture vessel. The culture vessel lid contained five ports, which held ground glass stoppers. Through these stoppers passed the tubes for gas, media inlet and outlet, sampling and pH control apparatus.

The gas supply to the culture vessel varied, depending on the conditions required. All of the gases used were supplied by BOC Ltd., and were delivered to the culture vessel along silicone



Figure 2.1 : Schematic representation of the chemostat employed in this study

A, culture vessel. B, culture vessel lid. C, fresh media reservoir. D, waste culture reservoir. E, connecting hood. F, membrane filter. G, burette. H, light bank. J, glass electrode. K, magnetic follower. L,N,O, air glass filters. M, magnetic stirrer. P,P1,P2, peristaltic pumps. Q,U, Hoffman clips. R, pH controller. S, thermometer.  
I, fresh media inlet. II, gas inlet. III, sample port. IV, effluent tube.



tubing (II), which had an ACRO 50 PTFE autoclavable filter (F) (Gelman Ltd.) inserted just above the glass sparger to ensure a sterile gas supply. The rate of gas flow to the culture vessel remained constant, the vessel being sparged at  $100\text{ cm}^3$  of gas/min., the flow rate being regulated by Gallenkamp flow meters (Gallenkamp Ltd.).

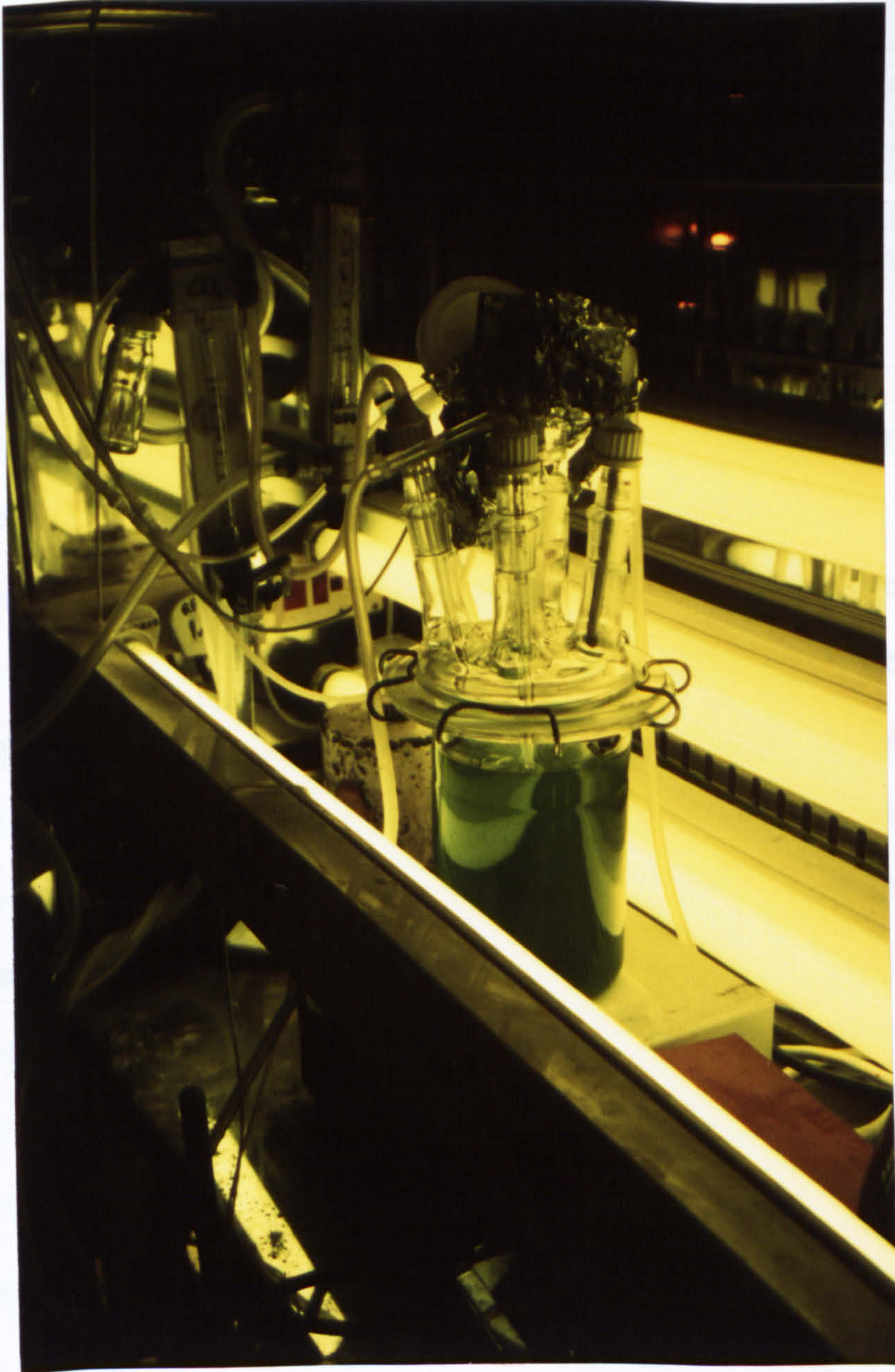
The fresh media reservoir (C) was a 10 l Pyrex glass vessel with a silicone rubber stopper through which two tubes passed. One was connected to an air glass filter (L), and was simply to prevent a back vacuum in the reservoir, whilst the other tube carried the sterile fresh medium. This was pumped along silicone tubing into the culture vessel through an inlet port (I) by a peristaltic pump (P) (Watson-Marlow Ltd.). A 'T'-piece (Portex, England), connected to a burette (G), was inserted into the silicone tubing between the peristaltic pump and the reservoir to measure media flow rates (see section 2.8.1.4).

Waste culture and effluent gases were removed from the culture vessel into the waste culture reservoir (D). Excess culture was removed in the following manner: as fresh medium entered the culture vessel, the volume increased, resulting in the end of the effluent tube (IV) being covered. This resulted in a temporary increase in pressure, resulting in the removal of some of the culture vessel contents along the effluent tube, which when uncovered again, allowed the escape of effluent gas.

A homogeneous suspension was maintained by stirring with a 5 cm magnetic follower (K) operated by a magnetic stirrer (M) (Stuart, England) which had its top plate covered with a layer



Figure 2.2 : Chemostat culture in operation





of polystyrene foam to insulate the culture from the heat it produced. In all experiments the same gentle rate of stirring was used, sufficiently rapid to ensure a homogeneous culture and minimise wall growth.

The temperature of the culture vessel was maintained at a constant 30°C. This was achieved by a combination of placing the chemostat in a controlled temperature room, and the heating effect of the lights surrounding the vessel, accuracy being maintained within + or - 1.0°C.

Light was provided by fluorescent warm white strip lights (Osram 65/80W). The culture vessel was surrounded by a bank of lights (H), providing an average light intensity of  $120 \mu\text{E m}^{-2} \text{s}^{-1}$ .

The pH of the culture vessel was maintained at 8.2, unless otherwise stated, regulated by the use of a LH505 pH controller (LH Fermentation). The pH controller (R) was connected to an autoclavable glass electrode (J), which passed through a silicone rubber stopper attached to the largest port. This same silicone rubber stopper also had two short lengths of glass tubing (3 mm diameter) passing through it, which were used to deliver either 0.1M NaOH or 0.1M HCl for pH correction.

Fresh medium reservoirs and waste culture reservoirs were periodically removed by aseptically disconnecting the relevant reservoir at the connecting hood (E) and attaching a fresh one.

## 2.9 Continuous-flow culture system operation

### 2.9.1 Autoclaving

The continuous-flow culture systems were autoclaved as three separate units. The culture vessel and waste culture reservoir were autoclaved at 15 psi for 15 minutes, and the fresh medium reservoir was autoclaved at 15 psi for 40 minutes.

The glass pH electrode was sterilized by placing in a 70% (v/v) solution of absolute ethanol for 30 minutes. Before placing in the culture vessel, the pH electrode was first washed with 1.0 l of sterile distilled water.

### 2.9.2 Inoculation

The culture vessel was inoculated aseptically through the inlet port (I) with 50 ml of stock culture. Initially the organism was grown as a batch culture, sparged with air. Once the culture had reached an  $OD_{750}$  greater than 1.0, the continuous-flow of fresh medium was started and the required  $CO_2$ -limited conditions imposed.

### 2.9.3 Sampling

Samples were removed from the culture vessel by the following procedure. The Hoffman clip (U) was closed to prevent the sparging gas from leaving the culture vessel, creating an immediate rise in pressure. Hoffman clip (Q) was then opened, and the rise in pressure in the culture vessel forced culture down the sample tube (III), where it was collected in a 25 ml universal bottle with a screw top. As soon as enough sample had



been collected Hoffman clip (U) was opened, Hoffman clip (Q) closed, the sample bottle removed and replaced with another one aseptically. Culture absorbance at  $OD_{750}$  (LKB Ultraspec II), biomass estimation, chlorophyll determination, inorganic carbon uptake and RuBisCO assay experiments were then performed on these samples.

#### 2.9.4 Measurement of flow-rate

Burette (G) was filled from the medium reservoir and by appropriate use of Hoffman clips, medium was pumped from the burette instead of the reservoir. The time taken to transfer a known volume of liquid from the burette to the culture vessel was determined and the flow rate calculated in terms of  $ml\ h^{-1}$ .

#### 2.9.5 Determination of a steady-state culture

To obtain a steady-state culture, the dilution rate was kept constant for a period of time greater than  $3 \times$  the culture doubling time ( $t_d$ ). Culture absorbance was then measured three times a day, and if it remained constant a steady state had been established

### 2.10 Inorganic carbon (Ci) uptake in unicellular cyanobacteria

#### 2.10.1 Vacuum filtration

This was the standard assay method for all of the organisms used in this study, whether grown in batch or continuous culture. Cells were harvested when in mid-logarithmic or a steady-state, centrifuged for 5 min at 5000 rpm at room temperature, and the

pellet resuspended in  $\text{CO}_2$ -free growth media (ph 7.4) or in a 10 mM buffer solution to approximately 1 mg dry wt  $\text{ml}^{-1}$ . The cells were then placed in an oxygen electrode (Hansatech, Kings Lynn) in the light at  $30^\circ\text{C}$ , unless stated otherwise, until cessation of  $\text{O}_2$  evolution. Aliquots (180  $\mu\text{l}$ ) were then placed in Eppendorf tubes and  $\text{NaH}^{14}\text{CO}_3$  (10  $\mu\text{Ci/mol}$ ) added to the required final concentration. This reaction mixture was then incubated under illumination ( $75 \mu\text{E m}^{-2} \text{s}^{-1}$ ) for the required time at either  $30^\circ\text{C}$  (freshwater cyanobacteria) or  $25^\circ\text{C}$  (saltwater cyanobacteria). The reaction was terminated by filtering the reaction mix onto pre-wetted Whatman GF/F filters, and washing the filters with 2 ml of 10  $\mu\text{M}$  unlabelled  $\text{NaHCO}_3$ , to get rid of any  $\text{NaH}^{14}\text{CO}_3$  still adhering to the outside of the cells. Duplicate experiments were run at each time point and  $\text{NaH}^{14}\text{CO}_3$  concentration, and the duplicate filters were treated as follows: after washing all filters were placed in scintillation vials. To one of a duplicate pair was immediately added 5 ml of Beckman EP scintillation fluid, and the radioisotope incorporation counted in an LKB mini-beta scintillation counter. This gave the total Ci incorporation. To the other was added 0.2 ml of 70% (v/v) perchloric acid, followed by incubation for 30 min over a  $\text{CO}_2$ -stream to remove acid-labile  $^{14}\text{Ci}$ . 5 ml of Beckman EP was then added and the vial treated as above. This gave the acid-stable (photosynthetic products) Ci incorporation. By subtracting this figure from the total, the Ci concentration in the 'internal pool' could be established. Ci incorporation was expressed in nmoles Ci incorporated per mg dry wt  $\text{ml}^{-1}$ .

### 2.11 Measurement of oxygen evolution

Cells were harvested by centrifugation at 5000g for 5 min in a Gallenkamp Labspin at room temperature. The pellet was resuspended in fresh growth medium at a concentration of approximately 1 mg dry wt ml<sup>-1</sup>, and placed in the chamber of an oxygen electrode (Hansatech Ltd, Kings Lynn), at 30°C and illuminated (light intensity = 75  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). After the cells had depleted their endogenous Ci reserves (shown by cessation of oxygen evolution), known amounts of sodium bicarbonate were added to the cell suspension and the rate of oxygen evolution in response to these additions recorded using a CR452 chart recorder (JJ Instruments).

### 2.12 RuBisCO assay

RuBisCO was measured by a slight modification of the permeabilized whole-cell assay used by Karagouni and Kelly (1989). Samples (containing 0.3-0.4 mg dry wt of cells) were taken directly from continuous-flow or batch culture, centrifuged in glass centrifuge tubes (4 ml), the supernatant removed, and the cell pellet resuspended in 0.2 ml cetyl-trimethylammonium bromide (CTAB). After 10 min 0.45 ml of the carboxylase assay mixture was added. This contained: Tris-HCl buffer pH 8.0 (77mM), MgCl<sub>2</sub> (25mM), reduced glutathione (2mM), and NaH<sup>14</sup>CO<sub>3</sub> (44mM) containing approximately 300 cpm <sup>14</sup>C per nmol. The suspension was then mixed thoroughly in a vortex,



and incubated at 30°C for 10 min. 0.15 ml of ribulose 1,5-bisphosphate (RuBP) was added to give an initial concentration of 3 mM and incubation continued. After 5, 10 and 15 min 0.2 ml aliquots were removed, and mixed with 0.1 ml 70% perchloric acid to discharge excess bicarbonate. Control incubations without RuBP were also run.  $^{14}\text{C}$  incorporation was measured by adding 10 ml Beckman EP Scintillant and counting in a Beckman LS7000 scintillation counter.

## 2.13 Isolation of cyanobacterial membranes

### 2.13.1 Cytoplasmic membranes, thylakoid membranes and soluble fractions

Cytoplasmic and thylakoid membranes were prepared by a modification of the method described by Omata and Ogawa (1986). Cells were grown up in 1, 2 or 10l volumes, harvested in mid-log phase (or when in a steady state in continuous-flow culture), centrifuged for 5 min at 8000 rpm in 500ml pots in a MSE Hi-Spin 21, and the pellets resuspended in 20ml of 5 mM Tes-NaOH buffer (pH 7.0) containing 0.6 M sucrose and 2mM EDTA.  $0.6 \text{ mg ml}^{-1}$  lysozyme was added and the suspension incubated at 30°C for 2 hours. The lysozyme-treated cells were then centrifuged at 8000 rpm for 5 min, washed with 20 mM Tes-NaOH buffer (pH 7.0) containing 0.6 M sucrose, suspended in 10 ml of the same buffer, and then disrupted by two passages through an Aminco French pressure cell at 1000 lb/sq in. 1mM of both PMSF and  $\epsilon$ -amino caproic acid were added to the homogenate which was then centrifuged for 5 min at 5000 rpm in an MSE chillspin to remove



any unbroken cells. The supernatant was made up to a sucrose concentration of 50% (w/v) by adding 0.74 volume of 90% sucrose solution. A 17 ml aliquot was placed at the bottom of a 35 ml polyallomer centrifuge tube, overlaid with 8 ml of 39%, 3 ml of 30% and 7 ml of 10% sucrose solutions (w/v), and centrifuged at 26,000 rpm for 16 h at 4°C in a Beckman SW28 swinging bucket rotor, using a Beckman L8 ultracentrifuge. All the sucrose solutions were ice cold, and contained 10 mM Tes-NaOH buffer (pH 7.0), 10 mM NaCl and 5 mM EDTA. Cytoplasmic membranes formed a band in the 30% sucrose layer and thylakoid membranes at the interface between the 39% and 50% sucrose layers. Cytoplasmic and thylakoid membranes were withdrawn from the gradient, diluted 3-fold with 10 mM Tes-NaOH buffer (pH 7.0) containing 10 mM NaCl, and pelleted by centrifugation at 60,000 rpm for 1 h in an MSE 10 x 10 titanium rotor.

Soluble fractions were prepared from the 50% sucrose layer. After a 3-fold dilution in 10 mM Tes-NaOH buffer (pH 7.0) containing 10 mM NaCl, the sample was centrifuged at 60,000 rpm for 1 h in an MSE 10 x 10 titanium rotor. The supernatant from this centrifugation was used as the soluble fraction.

### 2.13.2 Cell Walls

A carotenoid containing cell wall fraction was isolated using the method described by Resch and Gibson (1983). Harvested cells (100 ml) were resuspended in 4 ml 10 mM HEPES buffer (pH 7.2) and disrupted by two passages through a French pressure cell (as in 2.13.1) Whole cells were removed by centrifugation for 5 min

at 5000 rpm in an MSE Chillspin. The supernatant was layered onto a discontinuous 50-85% (w/v) sucrose gradient in 17 ml polyallomer centrifuge tubes and centrifuged at 4°C for 16 h at 25,000 rpm in a Beckman SW27 rotor using a Beckman L8 ultracentrifuge. All the sucrose solutions contained 10 mM HEPES (pH 7.2). The sucrose gradient was fractionated, the cell wall fraction being located in the 75-80% sucrose layer.

## 2.14 Quantitative analysis of membrane fractions

### 2.14.1 BioRad protein assay

This method was described by Bradford (1976). A micro-assay was routinely used. 0.8 ml of appropriately diluted sample was added to 0.2 ml dye reagent concentrate, gently mixed by inversion and the OD<sub>595</sub> read against a reagent blank after 10 minutes. The standard curve was prepared with 0.8 ml samples of bovine serum albumin (BSA) at five concentrations from 1-25 ug/ml.

### 2.14.2 Lowry protein assay

This method, using Folin phenol reagent was performed on boiled membrane fractions (5 min, 100°C), as described by Lowry et al (1951)

### 2.14.3 Chlorophyll determination

A known volume of cells (between 0.5-5.0 ml) was pelleted in an Eppendorf centrifuge, the supernatant removed, and the pellet resuspended in 2 ml of methanol. The sample was then wrapped in foil to prevent any light reaching it, and incubated for 10

minutes at 80°C in a preheated water bath. After cooling, the extinction at OD<sub>445</sub> was measured against a methanol blank.

## 2.15 Qualitative analysis of membrane fractions

### 2.15.1 Absorption spectrophotometry

Absorption spectra of samples were measured in a dual beam Pye Unicam SP8-200 spectrophotometer at room temperature in quartz cuvettes. The machine was zeroed to the appropriate sucrose solution and samples scanned from 250-700 nm. A scan speed of 1 nm/sec and a bandwidth of 1 nm was used with the chart speed set at 20 sec/cm.

### 2.15.2 Polyacrylamide gel electrophoresis (PAGE)

This technique was routinely used for the analysis of polypeptides from membrane preparations and also for the analysis of phosphopolypeptides in cell extracts and from *in vitro* kinase assays (see 2.16).

#### 2.15.2.1 One dimensional exponential gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of polypeptides was routinely carried out using the method of Kelly (1985). 10-30% (w/v) exponential gradient gels were cast using the apparatus described by Porter (1984). Plates used were either 19 x 17 cm BioRad glass plates with 1.5 mm spacers run on BioRad vertical gel apparatus, or more commonly 25 x 20 cm glass plates with 2 mm thick spacers. These gels gave good resolution and reproducible results. Gels



were run at 20 mA constant current at 4°C overnight (16 hours) in perspex tanks.

The stock solutions used were as follows:

60% (w/v) high bis-acrylamide, acrylamide

acrylamide (Fisons).....60.0g

bisacrylamide (Kodak).....1.6g

Acrylamide and bis-acrylamide were dissolved in 40 ml of warm distilled water, and the volume made up to 100 ml.

60% (w/v) low bis-acrylamide, acrylamide

acrylamide.....60.0g

bisacrylamide.....0.3g

Made up as above.

10% (w/v) stacking gel acrylamide

acrylamide.....10.0g

bisacrylamide.....0.5g

Lower gel buffer

Tris base.....36.6g

The pH was adjusted to 8.8 with HCl and the volume made up to 100 ml with distilled water

Stacking gel buffer

Tris base.....5.98g

The pH was adjusted to 6.8 with HCl and the volume made up to 100 ml with distilled water.

Running buffer stock (10x conc.)

Trizma base (25 mM final).....60.4g

Glycine (192 mM final).....288.0g

Sodium dodecyl sulphate (0.1% (w/v) final)....20.0g

Made up to 2 l with distilled water.

The composition of the various components needed to make the 10-30% exponential gradient polyacrylamide gel is given in Table 2.4. The 30% acrylamide was poured into a 25 ml glass scintillation vial containing a magnetic stirrer bar. The top of this scintillation tube was sealed with a Suba seal, which was penetrated by two 19G hypodermic needles (Sabre International Products Ltd.). One needle just penetrated the Suba seal (the inlet needle), whilst the other penetrated into the 30% acrylamide solution (the outlet needle). Both needles had a length of silicone rubber tubing attached to them. That from the outlet needle was attached to the back plate of the assembled casting plates, whilst that from the inlet needle passed through a peristaltic pump and into the 10% acrylamide solution, contained in a conical flask. The ammonium persulphate (AMPS), made up fresh, and TEMED were added to the 10% acrylamide and the peristaltic pump started. When the 10% acrylamide had nearly reached the inlet needle, the TEMED and AMPS were added to the 30% acrylamide and the Suba seal inserted in place, thus forcing a few drops of the 30% gel solution through the outlet needle and into the casting plate assembly. The stirrer speed was set

to 600 rpm and the peristaltic pump to 3 ml min<sup>-1</sup>. This system with a constant volume in the mixing vial being diluted by a larger volume of the lower percentage gel solution results in an exponential gel. The 10-30% part of the gel was known as the resolving gel and was poured until it was 4 cm from the top of the gel plate. The surface was overlayed with a small volume of water saturated butan-2-ol and the gel left for 3 hours to set. After polymerisation all traces of the butan-2-ol were removed by washing with distilled water, and the stacking gel layered on top. Immediately after pouring, a teflon comb with the appropriate number of well slots was inserted into the stacking gel, which was then allowed to set. The teflon comb was then removed and the gel transferred to the gel tank for running.

Protein samples were denatured by the addition of sample buffer and heated for five minutes in a boiling water bath, unless otherwise stated. The composition of sample buffer stock (2x conc.) is given in Table 2.4. SDS-PAGE low molecular weight markers (BioRad Laboratories) were run routinely on each gel. They comprised (molecular weights in brackets) phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and  $\alpha$ -lactalbumin (14,400).

#### 2.15.2.2 One dimensional non-denaturing polyacrylamide gels

These gels were used for the analysis of native proteins. Three additional stock items were required to those described above:



25% (w/v) high bis-acrylamide, acrylamide

acrylamide.....25.00g

bis-acrylamide.....1.25g

Both were dissolved in 40 ml warm distilled water, and the volume then made up to 100 ml.

30% (w/v) low bis-acrylamide, acrylamide

acrylamide.....30.00g

bis-acrylamide.....0.15g

Made up as above

Non denaturing gel running buffer

glycine.....43.20g

Tris.....9.00g

Made up to 1.5 l with distilled water. Gel running buffer was freshly made each time a non-denaturing gel was run.

The non-denaturing gel system used was a 4-15% gradient, the contents of which are described below.

4% (w/v) acrylamide solution

25% (w/v) acrylamide stock.....9.2 ml

lower gel buffer (2.15.2.1)..... 7.5 ml

distilled water.....40.8 ml

15% (w/v) acrylamide solution

30% (w/v) acrylamide stock.....10.0 ml

lower gel buffer.....2.6 ml

30% (v/v) glycerol.....7.4 ml

Polymerisation was initiated by the addition of 12.2ul TEMED and 104.5 ul 10% (w/v) AMPS to the 4% acrylamide solution and 3.0 ul TEMED and 30 ul 10% (w/v) AMPS to the 15% acrylamide solution.

The gradient was poured using the same technique and apparatus described in 2.15.2.1. The only difference was that no stacking gel was used, the resolving gel was poured to the top of the casting plates and a well comb immediatly inserted. After the gel had set (usually within three hours), the comb was removed and the gel placed in a gel tank in the cold room. The gel was then run overnight at 18 mA, using the running buffer described in this section.

2.15.2.3 Polyacrylamide gel staining2.15.2.3.1 Coomassie blue staining

The staining solution contained:

PAGE Blue 83 (Biorad).....2.5 g

methanol.....500 ml

glacial acetic acid.....70 ml

distilled water.....430 ml

Final volume 1 l. The PAGE Blue 83 was first dissolved in the methanol before the addition of the acetic acid and distilled water. The gel was stained by soaking in the Coomassie solution overnight. After staining the gel was destained in 45% (v/v)

Table 2.4    Composition of 10-30% SDS-PAGE resolving gel, stacking gel and sample buffer

30% gel (20 ml)		10% gel (50 ml)	
Low bisacryl stock	10.0 ml	High bisacryl stock	8.3 ml
75% (w/v) glycerol	7.3 ml	distilled water	34.9 ml
lower gel buffer	2.5 ml	lower gel buffer	6.25 ml
10% SDS	0.2 ml	10% SDS	0.5 ml
TEMED	0.004 ml	TEMED	0.01 ml
AMPS 10% (w/v)	0.04 ml	AMPS 10% (w/v)	0.1 ml

4% stacking gel		2x sample buffer stock	
stacking gel acryl	4.0 ml	stacking gel buffer	2.5 ml
distilled H <sub>2</sub> O	3.4 ml	distilled H <sub>2</sub> O	0.5 ml
stacking gel buffer	2.4 ml	glycerol	2.0 ml
10% (w/v) SDS	0.1 ml	10% (w/v) SDS	4.0 ml
TEMED	0.005 ml	B-mercaptoethanol	0.1 ml
AMPS 10% (w/v)	0.1 ml	bromophenol blue	0.1 ml



methanol, 10% (v/v) glacial acetic acid.

This procedure is insensitive, requiring about 100 ug total cell protein per track (0.2-0.5 ug protein in a sharp band).

This was the preferred staining method for phosphopolypeptides, where normally only the non-radioactive standards have to be visualised, the phosphopolypeptides showing after autoradiography, and also because silver staining resulted in excessive quenching of the radioisotope emissions. In this case a simplified staining procedure was adopted, the gel was soaked in the staining solution for two hours and then transferred to the TCA phosphoprotein protocol (see 2.16.3). The heat treatment in TCA was found to be an extremely effective destain.

#### 2.15.2.3.2 Silver staining

The method of Wray et al. (1981) was routinely used for analysis of cyanobacterial membrane proteins. This detects proteins at the nanogram level, is rapid, reproducible, requires few reagents and little preparation. After electrophoresis the gel was soaked in 50% (v/v) methanol for at least 5-6 hours, the soaking solution being changed 3 or 4 times during this period. The gel was then placed in the staining solution for 15 minutes. The staining solution consisted of two solutions:

##### solution A

3.2 g silver nitrate in  
16 ml distilled water

##### solution B

84 ml 0.36% (w/v) NaOH  
5.6 ml 14.8 M ammonium hydroxide

The stain solution was freshly prepared by adding solution A dropwise to solution B with stirring, and made up to 400 ml with distilled water. After staining, the gel was washed twice in distilled water, before being transferred to the developing solution (see below) until the appearance of the appropriate bands on the gel, after which the developing reaction was stopped by the addition of a solution containing 45% methanol and 10% glacial acetic acid.

Silver stain developing solution

2.5 ml 1% (w/v) citric acid

0.4 ml 38% (v/v) formaldehyde solution

Made up to 500 ml with distilled water.

2.15.3 Western blotting

Proteins separated by PAGE were transferred to nitrocellulose using a BioRad transblot system at 300 mA for 3 hr in buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol. Proteins transferred to nitrocellulose were visualised using Ponceau S (0.5% w/v in 5% TCA). The filter was left in the dye solution for 10 min with shaking, and unbound dye removed by washing with water. To remove all remaining dye, filters were transferred to 0.02 M sodium phosphate pH 7.3 (PBS) solution. The Amersham peroxidase conjugated goat anti-rabbit secondary antibody detection system was then used for immunoscreening. Following the removal of Ponceau S with PBS, filters were blocked in 50 ml 0.14 M NaCl, 0.02 M PBS containing 3% dried

milk powder (Sainsbury's, U.K. Ltd.) with shaking for 1 hr at room temperature. Primary antibody was added to the appropriate dilution (1:1000) and filters incubated for 1 hr. After five 10 min washes in PBS containing 1% Triton X-100, filters were transferred to a solution of 1% Triton X-100 in PBS containing peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:5000) and left shaking for 1 hr. After five 10 min washes in PBS, filters were transferred to a solution containing 160 ml 10 mM Tris pH 8.0, and 1 ml O-dianisidine solution added (14 mg O-dianisidine in 1 ml methanol). 50  $\mu$ l hydrogen peroxide was then added to initiate the staining reaction.

## 2.16 $^{32}$ P-orthophosphate incorporation into unicellular cyanobacteria

### 2.16.1 in vivo incorporation

Radioisotope incorporation was determined under a variety of growth conditions using acid-free  $^{32}$ P-orthophosphate (Amersham or NEN) in aqueous solution with a radioactive concentration of 10 mCi ml<sup>-1</sup>. This was routinely added to a final radioactive concentration of 10  $\mu$ Ci ml<sup>-1</sup> to cultures in early exponential phase for continuous labelling experiments. In continuous labelling experiments, unless stated otherwise, after addition of the radioisotope and two generations growth, the cells were transferred to another carbon regime, and at the required time intervals 4 ml samples were withdrawn from the culture. These were placed in safe-lock Eppendorf tubes and pelleted in an Eppendorf microfuge. The supernatant was discarded, the pellet



washed twice with ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM  $\text{MgCl}_2$ , and then stored until required at  $-20^\circ\text{C}$ , in either 0.4 ml of the same buffer for non-denaturing gels or 0.4 ml sample buffer (see Table 2.4) for denaturing gels.

#### 2.16.2 in vitro incorporation

This was performed using a cell-free extract of the cyanobacterial species required, prepared by pelleting a sample of exponentially growing cells, washing twice in 2x kinase buffer (100 mM Tris-HCl pH 7.5, 20mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 5 mM  $\beta$ -mercaptoethanol), and in the same medium disrupting the cells by two passages through a French pressure cell at 24,000 psi. Unbroken cells and cell debris were removed by centrifugation for 10 min in an Eppendorf centrifuge, the supernatant diluted to a protein concentration of  $1 \text{ mg/ml}^{-1}$  and stored at  $-20^\circ\text{C}$  until required.

The reaction mixture for *in vitro* phosphorylation contained the following:

25.0 ul cell extract (in 2x kinase buffer)

7.5 ul distilled water

12.5 ul test substance

5.0 ul gamma labelled-ATP (2.5  $\mu\text{Ci/ul}$ ) (ICN Ltd)

Total reaction volume 50 ul. The reaction was started by the addition of the gamma labelled-ATP, incubations taking place at  $30^\circ\text{C}$  for 30 minutes. Reactions were terminated by the addition of an equal volume of 2x sample buffer and boiling at  $100^\circ\text{C}$  for 5 minutes.

### 2.16.3 Phosphoprotein gel treatment

As with all gels it was important that each track contained the same loading of protein, the protein concentration of each sample being determined using the BioRad microassay (see 2.14.1).

The visualisation of phosphoproteins on polyacrylamide gels was complicated by many factors, several of which could be overcome using an adaptation of the hot TCA treatment for polyacrylamide gels developed by Bhorjee and Pederson (1976). The gel was placed in a Pyrex dish with 800 ml 16% (w/v) TCA, 35% (v/v) methanol. This was then incubated for 40 minutes in a water bath that had been previously heated to 95°C. After incubation the dish was immersed in a sink of cold water for cooling. After cooling the gel was then placed in washing buffer (5% (w/v) TCA, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 35% (v/v) methanol), and washed for 24 hours with at least three changes of buffer, before being dried down and autoradiographed.

In early experiments 20ug/ml<sup>-1</sup> of DNase I and RNase A were incubated for 30 minutes with both *in vitro* and *in vivo* samples, before they were frozen, however the hot TCA treatment described above proved very effective at removing <sup>32</sup>P-labelled nucleic acids, and so this pre-treatment was abandoned.

While it was theoretically possible to stain gels with coomassie blue after the hot TCA treatment (Mannai and Cozzone, 1979a), it was found that the staining was poor and produced a much higher background, thus as a matter of routine gels were stained prior

to hot TCA treatment, which as already mentioned, was a very effective destain.

#### 2.16.4 Gel drying and autoradiography.

Gels were mounted on a double layer of Whatman 3MM chromatography paper and dried under vacuum at 80°C on a BioRad dual temperature slab gel dryer.

For the autoradiography of <sup>32</sup>P-labelled gels, the dried gels were placed in an X-ray cassette (X-Ray accessories Ltd, Bushey, Herts) with Fuji RX X-Ray film sandwiched between two Dupont lightning-plus intensifier screens. The film was exposed at -70°C, initially for 24 hours, and then a second exposure was taken, the time depending on the intensity of the bands produced by the 24 hour exposure.

Autoradiographs were developed in Kodak LX-24 X-Ray developer and fixed in Kodak FX-40 X-Ray fixer.

### 2.17 General molecular biological techniques

#### 2.17.1 Restriction endonuclease digestion

The low, medium and high salt buffers used were obtained from Amersham at 10x concentration. After adding 1 ul of 10x restriction buffer for every 9 ul DNA, restriction enzyme was added and the digestion carried out at 37°C for at least 1 hr, unless stated otherwise. Spermidine (4 mM final concentration) was added to restriction digests of plasmid DNA prepared by the small scale method. Restrictions were terminated by placing the



samples on ice, and any samples not used immediately were frozen at  $-20^{\circ}\text{C}$ .

#### 2.17.2 Agarose gel electrophoresis

0.7% (w/v) horizontal agarose slab gels were routinely used. These were prepared by boiling agarose (Sigma, Type 1 low EEO) in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). The agarose was cooled to around  $50^{\circ}\text{C}$  before pouring. DNA samples were prepared for loading by adding 0.2 volume of DNA loading buffer (see Table 2.5). Electrophoresis was carried out with the gel completely submerged in agarose gel electrophoresis buffer (1x TBE) and run at 80 mA for 1 hr (mini-gel system) or at 30 mA overnight. DNA was visualised by transillumination with short wavelength UV light (260 nm) and photographed using Polaroid 665 film.

#### 2.17.3 DNA restriction fragment isolation from agarose gels

Electroelution of DNA fragments from agarose gels was performed using an IBI electroelution chamber. The protocol used was that described by the manufacturer.

#### 2.17.4 Dephosphorylation of plasmid DNA

This was performed to prevent self-ligation of plasmid (or vector) DNA. To already restricted plasmid DNA, a 10% volume of 1 mM  $\text{ZnCl}_2$ , 10 mM  $\text{MgCl}_2$ ; a 10% volume 0.5 M glycine pH 9.4 (with NaOH) and 1-2 units of alkaline phosphatase (Boehringer

Table 2.5 Composition of DNA loading buffer per 10 ml

glycerol	2.0 ml
1 M Tris pH 8.0	0.1 ml
0.1 M EDTA	1.0 ml
distilled water	6.9 ml
agarose	20 mg
bromophenol blue	10 mg
xylene cyanol	10 mg
Orange G	10 mg

Mannheim) was added. This was incubated at 37°C for 1 hr, the DNA extracted with phenol:chloroform and ethanol precipitated (see below).

#### 2.17.5 Phenol:chloroform DNA extraction

Phenol:chloroform was prepared by dissolving 100 g phenol and 100 mg 8-hydroxyquinilone in 100 ml chloroform : 4 ml isoamyl alcohol. This was equilibrated by shaking the mixture with two changes each of both 0.2 volume 1M Tris (pH 8.0) and 0.2 volume TE buffer ( 10 mM Tris, 1 mM EDTA pH 8.0), before storage at 4°C.

DNA samples were mixed with an equal volume of phenol:chloroform until an emulsion formed. The two phases were separated by centrifugation for 5 min in an Eppendorf centrifuge, and the upper aqueous layer removed. Two further extractions with chloroform:isoamyl alcohol (24:1) were carried out, to remove any remaining phenol. DNA was then recovered by ethanol precipitation.

#### 2.17.6 Ethanol precipitation

To the DNA solution 0.1 volumes 3 M sodium acetate pH 5.6 and 2 volumes of ethanol (-20°C) were added, mixed by vortexing and chilled at -20°C overnight. The DNA was precipitated by centrifugation for 15 min in an Eppendorf microfuge at 4°C, the supernatant discarded and the DNA pellet dried under vacuum. The pellet was resuspended in 50ul T/0.1E buffer (10 mM Tris, 0.25 mM EDTA pH 8.0) and used as required or stored at -20°C.



### 2.17.7 DNA ligation

Restricted plasmid DNA (dephosphorylated) and isolated DNA fragments were mixed in appropriate volumes of T/0.1E. To subclone fragments, a 4:1 fragment:vector ratio was used, as described by Legerski and Robberson (1985). For library constructions, vector DNA was present in large excess. The ligation mixture was heated at 65°C for 10 min, and allowed to reanneal slowly on ice for 1 hr. After adding appropriate amounts of 10x ligation buffer (0.5 M Tris pH 7.4, 0.1 M MgCl<sub>2</sub>, 0.1 M dithiothreitol, 10 mM spermidine, 1 mg/ml BSA, 10 mM ATP) and T4 DNA ligase, the mixture was incubated at 15°C for 16 hr.

### 2.18 Small scale plasmid isolation from cyanobacteria

Isolation of plasmid DNA followed the procedure of van den Hondel et al. (1979), as modified by Reaston et al (1980). BG-11 (100 ml) was inoculated with 0.5 ml of a well grown culture and incubated for three days at 30°C. The cells were pelleted, washed once with 10 ml SE-solution (0.12 M NaCl, 0.05 M EDTA) and then with 10 ml lysis buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose). The cells were then resuspended in 0.5 ml lysis buffer and 0.25 ml lysozyme (10 mg/ml) added. After incubation for 1 hr at 37°C, the cells were lysed by the addition of 0.25 ml 10% SDS. The lysed cells were then left at 37°C for 1 hr, 0.25 ml 5M NaCl added, the solution mixed gently and then left overnight at 4°C. The cell debris was removed by

centrifugation in an Eppendorf centrifuge for 30 min, and the DNA isolated by phenol:chloroform extraction and ethanol precipitation (see above)

#### 2.19 Chromosomal DNA isolation from cyanobacteria

Cyanobacterial chromosomal DNA extraction was based on the method described by Lind et al, (1985), as modified by Scanlan (1988).

A late-log phase culture (25 ml) was spun in an MSE multex centrifuge at 5,000 rpm for 10 min and resuspended in 0.5 ml 0.25 M Tris pH 8.0; 20% (w/v) sucrose containing lysozyme (10 mg/ml), and incubated for 1 hr at 37°C. 16  $\mu$ l 30% (v/v) sarkosyl and 20  $\mu$ l 5 mg/ml proteinase K was then added, and the cells incubated for 1 hr at 65°C. An equal volume of phenol:chloroform was added, mixed by vortexing and spun in an Eppendorf centrifuge for 4 min. The supernatant was dialysed overnight against TE buffer at 4°C. The DNA solution was then concentrated by ethanol precipitation.

#### 2.20 Large-scale plasmid isolation from *Escherichia coli*

The procedure of Clewell and Helinski (1970), for the isolation of multicopy-plasmids was followed. An *E.coli* culture (500 ml) grown in nutrient broth with the appropriate antibiotic added was harvested by centrifugation at 8000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 16.5 ml Tris-sucrose (0.05 M Tris, 0.73 M sucrose, pH 8.0) in Oakridge tubes. 5 ml lysozyme (10 mg ml<sup>-1</sup>) in 0.25 M Tris (pH 8.0) was

added, and the cells left on ice for 5 min. 4.5 ml 0.25 M EDTA pH 8.0 was then added, and the cells left on ice for a further 5 min, after which 18 ml lysis mix (500 ml lysis mix: 3.03 g Tris, 11.63 g EDTA, 10 g Brij 58, 2.0 g sodium deoxychlorate pH 8.0) was added. The tubes were then inverted until the solution went clear (sometimes alternate incubations at 42°C were required). The cleared lysate was then centrifuged for 15 min at 18,000 rpm in a Hi-spin 21 at 4°C, which pellets unlysed cells, cell debris and chromosomal DNA. 29 g caesium chloride was then added to 29 ml of the supernatant and allowed to dissolve. 3 ml Ethidium bromide (10 mg ml<sup>-1</sup>) was added, and the solution transferred to Beckman polyallomer heat sealed tubes. The preparation was spun for 16-18 hr in a VTi50 rotor (Beckman) at 45,000 rpm, 15°C. Plasmid DNA was visualized using a longwave UV light, and was removed through the side of the tube using a 21 gauge needle and 5 ml syringe. The DNA was extracted twice with water saturated butan-1-ol (which removes ethidium bromide), and to 3 ml DNA solution, 1.2 ml 1% sarcosyl, 1.2 ml 3 M sodium acetate pH 5.6, 6.6 ml T/0.1E and 24 ml 100% ethanol were added. After mixing by inversion, the tubes were stored at -20°C overnight. The DNA was precipitated by centrifugation at 18,000 rpm for 40 min in a Hi-spin 21 at 4°C. The supernatant was removed using a pasteur pipette, and the pellet dried in a vacuum. Once dry, the pellet was resuspended in 0.5 ml T/0.1E and the plasmid DNA stored at -20°C.



## 2.21 Transformation of cyanobacteria

Transformation of *Synechococcus* R2 and *Synechococcus* R2-Spc was performed as described by Kuhleimeier et al. (1981). A 2-3 day old culture, grown with fast shaking, was pelleted, washed once in BG-11, and resuspended to give a final concentration of  $5 \times 10^8$  cells/ml. DNA (10ul) was added to 0.2 ml of cells in clear polypropylene tubes and incubated in the light for 30 min. Cells were plated on BG-11 agar under non-selective conditions and incubated in the light for 16-18 hr. Antibiotic (0.5 ml) was added underneath the agar at the desired concentration (see Table 2.6), and the plates reincubated in the light. Transformants were detected after 5-6 days growth, transformant colonies being restreaked on selective media.

## 2.22 Transformation of *Escherichia coli*

An overnight culture of *E.coli* MC1061 was diluted 1:25 in SOB medium (see section 2.6) in a 250 ml flask and incubated at 37°C in an orbital shaker (250 rpm) until the  $OD_{550} = 0.35$ . Transformation with plasmid DNA was then carried out by the calcium chloride procedure, as described by Maniatis et al. (1982), using Sigma grade I calcium chloride. This procedure produced  $10^4$ - $10^7$  transformants per ug pBR322 DNA.

Table 2.6 Minimum inhibitory concentrations of antibiotics used for selection of transformants in *Synechococcus* R2-SPc and *Escherichia coli* (in  $\mu\text{g}/\text{ml}^{-1}$ )

<u>Strain</u>	<u>chloramphenicol</u>	<u>ampicillin</u>	<u>Kanamycin</u>
<i>E.coli</i>	30	50	25
<i>Syn.R2</i>	7.5	1.0	25

## Chapter 3

Inorganic carbon uptake in cyanobacteria grown  
under different carbon regimes in batch culture



### 3.1 Introduction

As already mentioned in chapter 1, cyanobacteria are capable of actively transporting and concentrating bicarbonate internally in response to changes in their carbon regime. The process has been studied in detail in numerous species of cyanobacteria over the past decade (see reviews by Badger, 1987; Miller, 1990; Miller et al., 1990) and in all so far studied, the mechanism has been present.

The major aim of the work presented in this chapter was to build upon the work already carried out in cyanobacteria by assessing the  $C_i$  concentrating abilities of the various cyanobacteria listed in Table 2.1a. Every strain listed has been "quantitatively" assessed for its  $C_i$  concentrating ability, and has also been subjected to certain environmental changes during this assessment, namely changes in the external pH, to assess the favoured carbon species for the transport process. In addition *Synechocystis* PCC6803 has also been subjected to more stringent analysis in an attempt to study the  $C_i$  concentrating mechanism in response to the organism's growth rate and nutritional factors apart from the  $CO_2$  regime the organism was grown under. To this end, the effect of chemoheterotrophic and photoheterotrophic growth on the  $C_i$  concentrating mechanism in *Synechocystis* PCC6803 has also been studied.

An attempt has been made to correlate the ability/inability to concentrate inorganic carbon with the RuBisCO activity of the organism, and relate both of these factors to the growth of the organism in its natural environment.

At the outset of this study, silicone oil centrifugation was the method employed by most laboratories for analysing the Ci concentrating mechanism and although still popular, it has recently been superceded by methods that allow *in situ* analysis of the Ci concentrating mechanism, in particular membrane inlet mass spectroscopy (MIMS) (see Miller et al., 1989) which allows direct observation of the disequilibrium between the various carbon species, and hence the ability to distinguish between CO<sub>2</sub> and HCO<sub>3</sub> transport. The silicone oil centrifugation method suffers from being very time consuming, and in addition was found to give inconsistent results, and other aims were to develop a quicker, more consistent method for analysing Ci transport in cyanobacteria.

### 3.2 Materials and methods

Vacuum filtration, the standard method used in this study for analysing the  $\text{Ci}$  concentrating abilities of cyanobacteria has been described in section 2.10.1, and below are listed the other methods which were used solely with *Synechocystis* PCC6803 and *Synechococcus* PCC7942.

#### 3.2.1 Silicone oil centrifugation

An adaptation of the method of Miller and Coleman (1980) was followed. Assays were performed in 400  $\mu\text{l}$  Eppendorf microfuge tubes at  $30^\circ\text{C}$  in the light. 100  $\mu\text{l}$  of 2M NaOH (the terminating solution) was placed in the bottom of the Eppendorf tube, followed by 50  $\mu\text{l}$  of a silicone oil mixture consisting of 55% AR20 : 45% AR200 (v/v) (Wacker-chemie, Munich). The tubes were then given a 2 second spin in a Beckman microfuge II to compact the layers. A 180  $\mu\text{l}$  sample of exponentially growing cells, concentrated to approximately  $1 \text{ mg dry wt ml}^{-1}$ , and previously depleted of endogenous  $\text{Ci}$  (as shown by cessation of  $\text{O}_2$  evolution in an oxygen electrode) was placed on top.

20  $\mu\text{l}$  of a solution of  $\text{NaH}^{14}\text{CO}_3$  ( $10 \mu\text{Ci}/\mu\text{mol}$ ), diluted to give the required final concentrations, was then injected into the cell suspension, and incubated for the required time period at  $30^\circ\text{C}$  at a light intensity of  $75 \mu\text{E m}^{-2} \text{ s}^{-1}$ . After the designated incubation time, the reaction was terminated by a 10 second spin in a Beckman Microfuge II. The tubes were then immediately frozen in liquid nitrogen, and within 2 hr analysed in the following way ; The bottom of the Eppendorf tubes were cut off



at the interface of the terminating solution and the silicone oil layer whilst still frozen. The terminating solution was allowed to thaw, the pellet of cells resuspended in the terminating solution and transferred to a 0.5 ml Eppendorf tube. The bottom of the microfuge tube was then rinsed with another 100  $\mu$ l of 2 M NaOH to remove any remaining cells, and this was added to the original 100  $\mu$ l. This was then split into two equal portions. Half were placed in a plastic scintillation vial and 5 ml Beckman EP scintillation fluid added. Radioactive incorporation was determined by counting in an LKB mini-beta scintillation counter. The counts obtained represented the total Ci uptake by the cells. The remaining 100  $\mu$ l was acidified by adding an equal volume of 70% (v/v) perchloric acid. This was mixed thoroughly by vortexing, and placed in a CO<sub>2</sub> stream for 30 min, before radioisotope incorporation was determined as above. The counts obtained represented the Ci fixed into acid-insoluble photosynthetic products, and by subtracting this figure from the total Ci counts, Ci incorporation into the internal Ci pool could be determined.

### 3.2.2 Silicone oil centrifugation using dual-labelled cells

The same experimental format was used as in section 3.2.1, however the cells used were grown in BG-11, containing a 10<sup>-4</sup>M solution of <sup>3</sup>H-leucine (1  $\mu$ Ci/ $\mu$ mol).

### 3.3 Results and discussion

#### 3.3.1 Growth of unicellular cyanobacteria under different carbon regimes

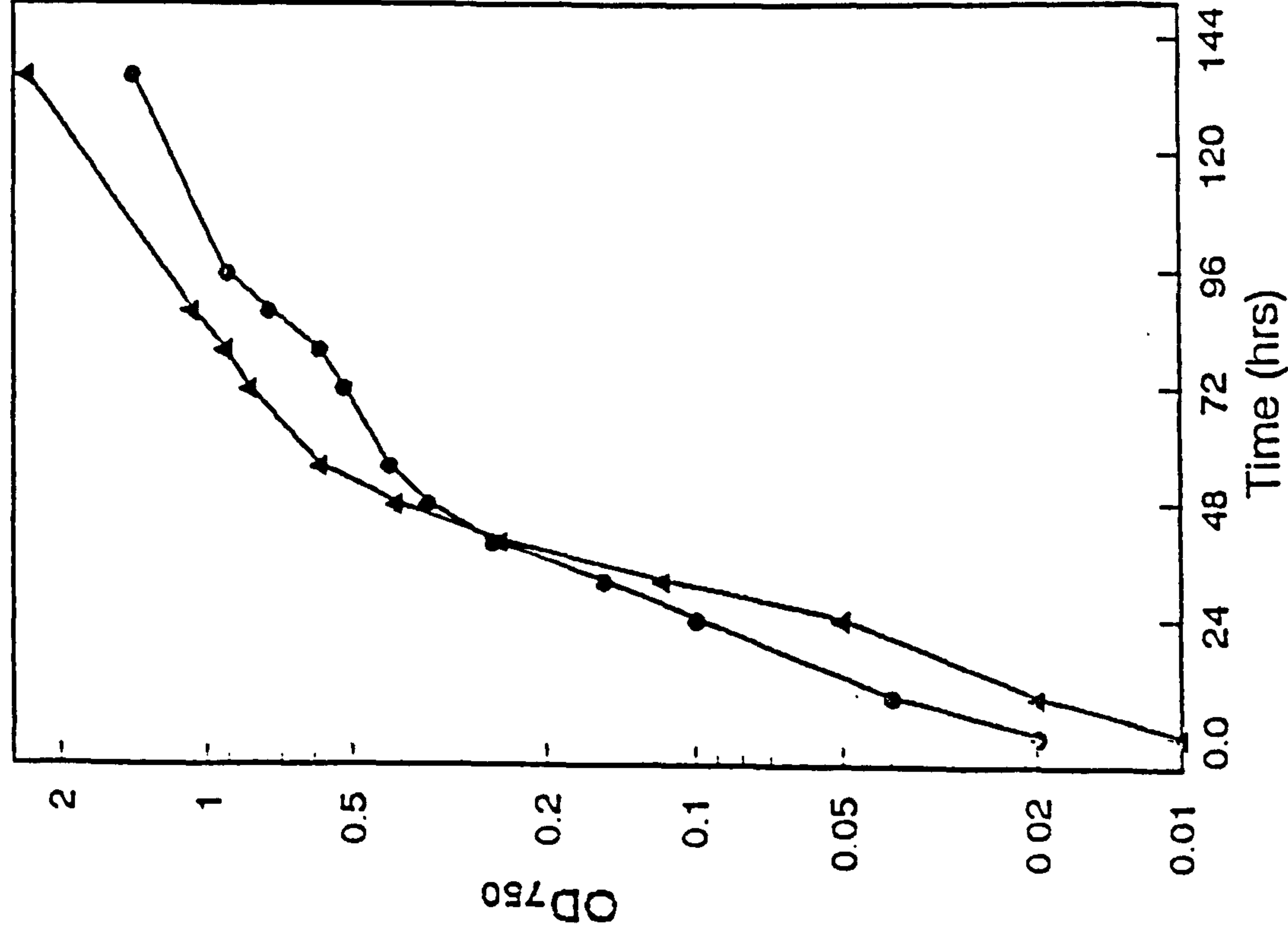
Typical batch culture growth curves for the majority of the organisms listed in Table 2.1a, grown under high and low CO<sub>2</sub> regimes are shown in Figs 3.1 through to 3.3.

In all the organisms studied growth rates were faster under high CO<sub>2</sub> as opposed to low CO<sub>2</sub> growth conditions. From present experimental evidence this is what we would expect, since with the exception of *Synechococcus* DC2, there is an active Ci uptake system in organisms grown under low CO<sub>2</sub> conditions (see Fig. 3.6 and section 3.3.3.5) which is very energy dependent (Raven and Lucas, 1985). These authors have calculated that operating such a mechanism to ensure a high CO<sub>2</sub>:O<sub>2</sub> ratio at the active site of RuBisCO may be energetically preferable to the costs of phosphoglycollate synthesis and its disposal via the photorespiratory carbon oxidation (PCO) cycle, but the capital costs of synthesizing a Ci concentrating mechanism, which can result in considerable changes to the cell boundaries (see Marcus et al., 1982) and the energy costs of running this system mean that a large proportion of the energy and CO<sub>2</sub> fixed will not be available for active growth. In contrast, in high CO<sub>2</sub>-grown cells, diffusion is sufficient to ensure a high enough CO<sub>2</sub>:O<sub>2</sub> ratio at the active site of RuBisCO to suppress the oxygenase reaction, and hence dispell the need for a Ci concentrating mechanism (see Raven and Lucas, 1985). In these cells, as more of the quantum energy and CO<sub>2</sub> fixed is available

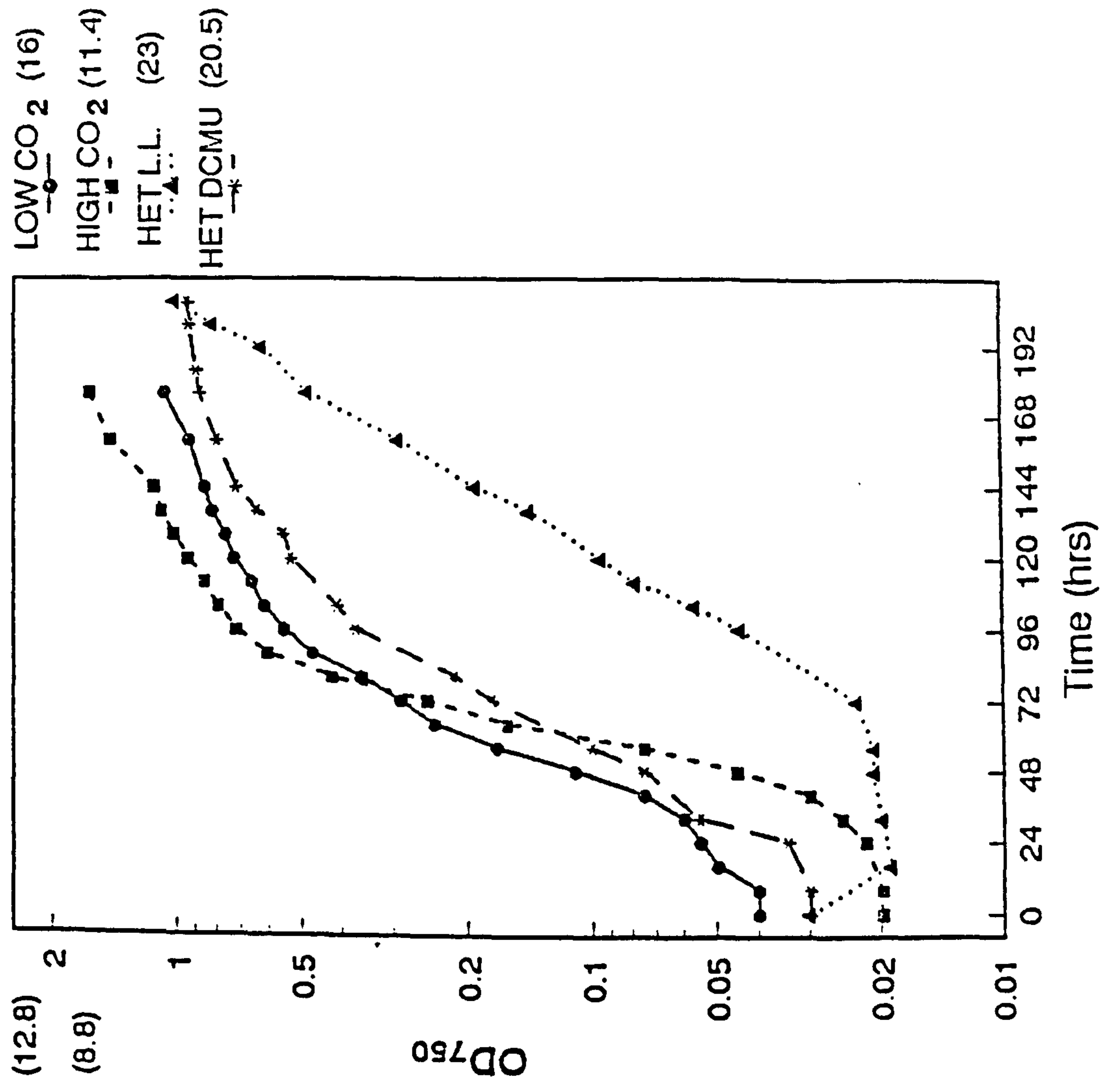
Figure 3.1 : Growth of *Synechococcus* PCC7942 and *Synechocystis* PCC6803 under different carbon regimes

HET L.L. ; Photoheterotrophically grown cells ( $5 \text{ uE m}^2\text{s}^{-1}$ ): HET DCMU ; Photoheterotrophically grown cells ( $35\text{-}40 \text{ uE m}^2\text{s}^{-1}$ ) + DCMU ( $10^{-5} \text{ M}$ ) . Figures in brackets represent doubling times of the organism.

#### *Synechococcus* PCC7942



#### *Synechocystis* PCC6803





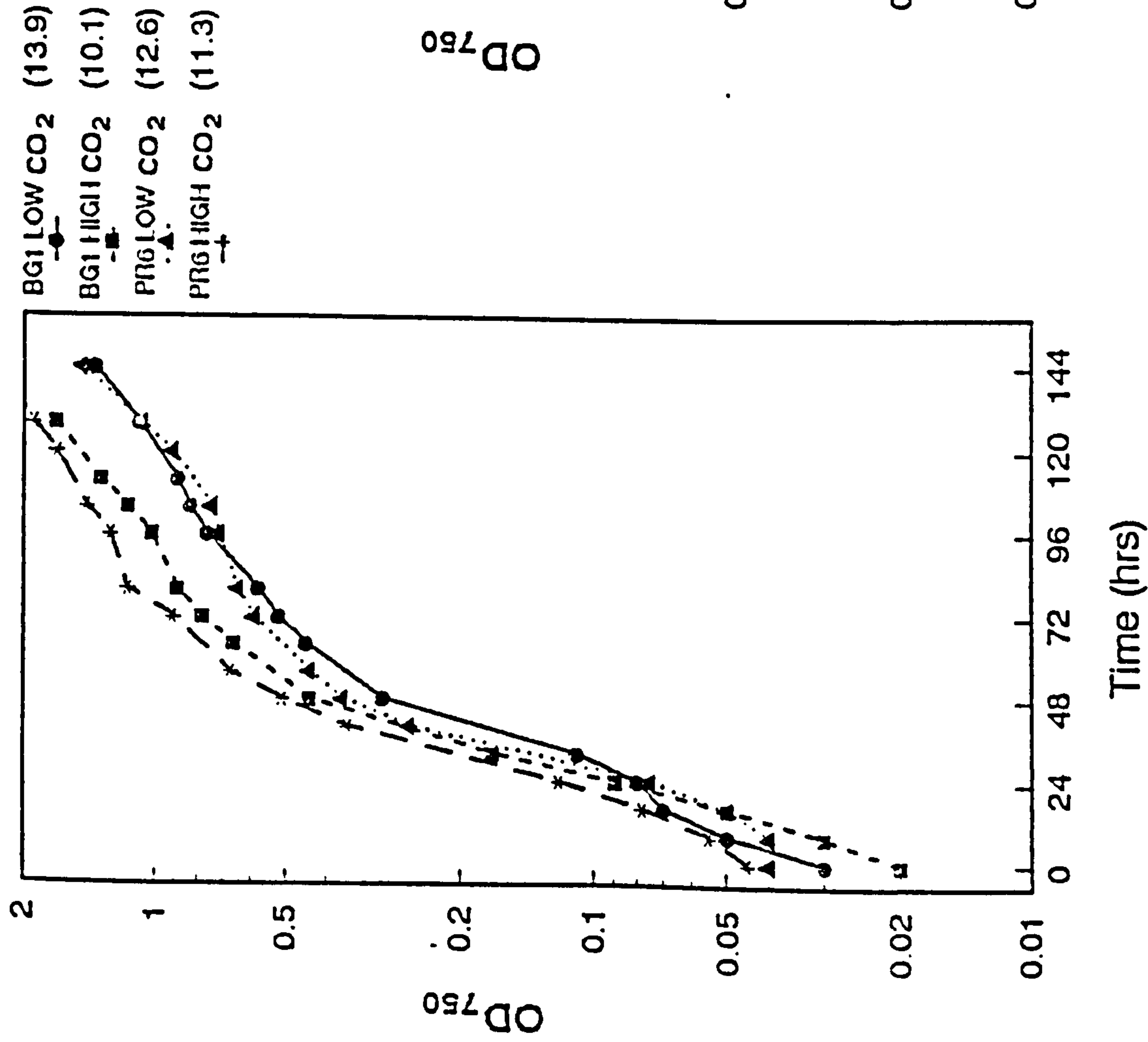
for active growth, growth rates are faster.

*Synechocystis* PCC6803 is also capable of photoheterotrophic growth using glucose as a carbon source (see Rippka et al., 1979), and in Fig. 3.1, growth rates were determined under two different photoheterotrophic conditions, growth at low light intensities ( $5 \text{ uE m}^{-2} \text{ s}^{-1}$ ) and under standard light intensities ( $35\text{--}40 \text{ uE m}^{-2} \text{ s}^{-1}$ ) with the addition of DCMU ( $10^{-5} \text{ M}$ ). DCMU at this concentration completely inhibits photoautotrophic growth in *Synechocystis* PCC6803 (data not shown). Under both of these conditions growth was considerably slower than under photoautotrophic conditions, with growth under low light conditions giving the slowest growth rates. As DCMU only inactivates photosystem II, ATP can still be generated through cyclic photophosphorylation. A preliminary study of the kinetics of the glucose uptake system in *Synechocystis* PCC6803 by Flores and Schmetterer (1986) indicated similar affinities to those shown by *Synechocystis* PCC6714, and it has been shown in this strain that uptake of glucose was more rapid in the light than the dark (Beauclerk and Smith, 1978). A combination of these two factors is the most likely reason for the faster growth rates seen in *Synechocystis* PCC6803 grown in the light. Although the light levels employed for photoheterotrophic growth under low light conditions would not support photoautotrophic growth (data not shown), strict heterotrophic growth on glucose in absolute darkness was not observed. As these light intensities are too low for active growth on  $\text{CO}_2$ , it suggests that the reason for the requirement of low light intensities under this growth

Figure 3.2 : Growth of *A. quadruplicatum* & *Synechocystis*  
sp. under high and low CO<sub>2</sub> regimes

Figures in brackets represent doubling times of the organism

*A. quadruplicatum* BG1 & PR6



*Synechocystis* PCC6714 & PCC6308

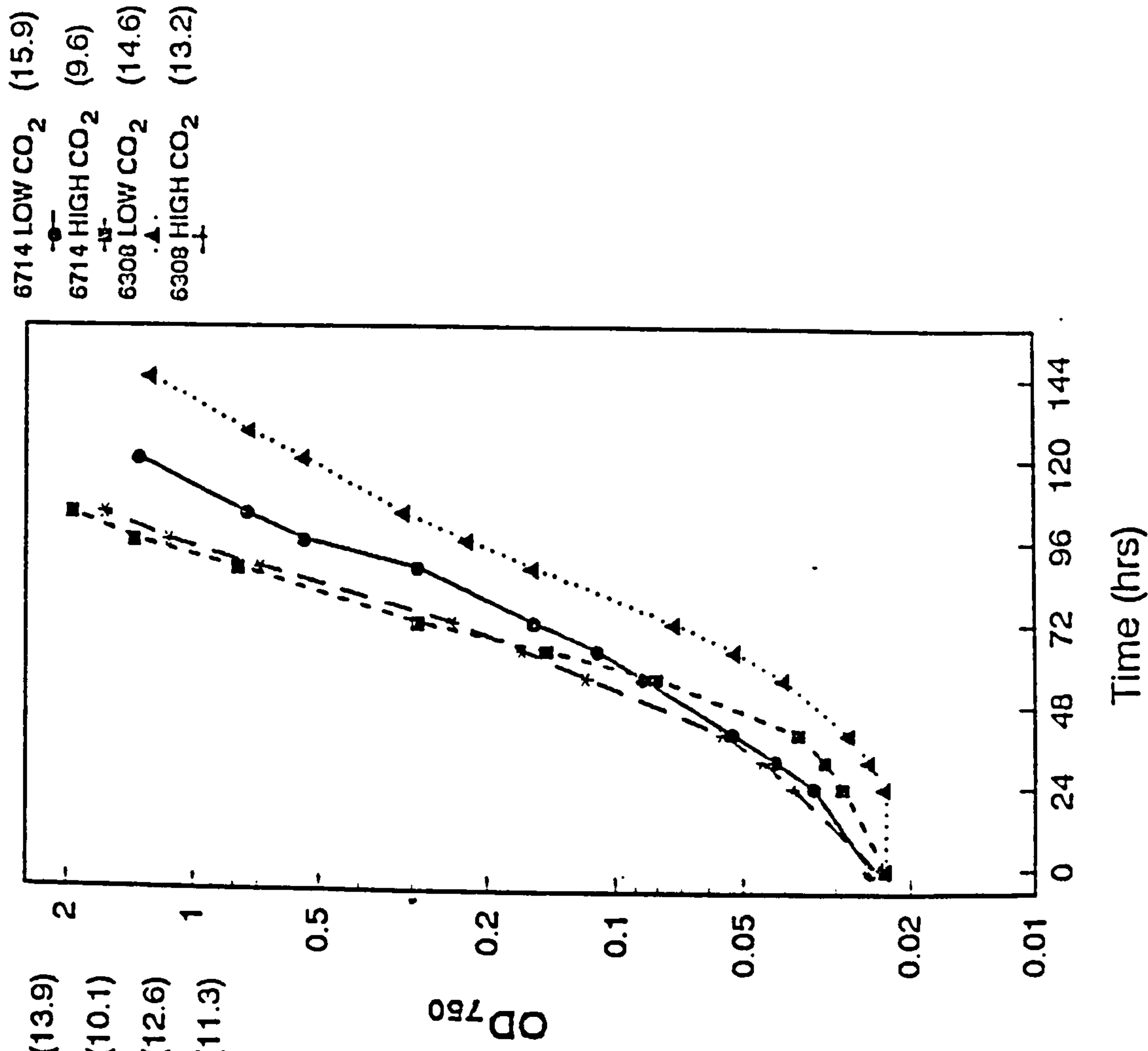
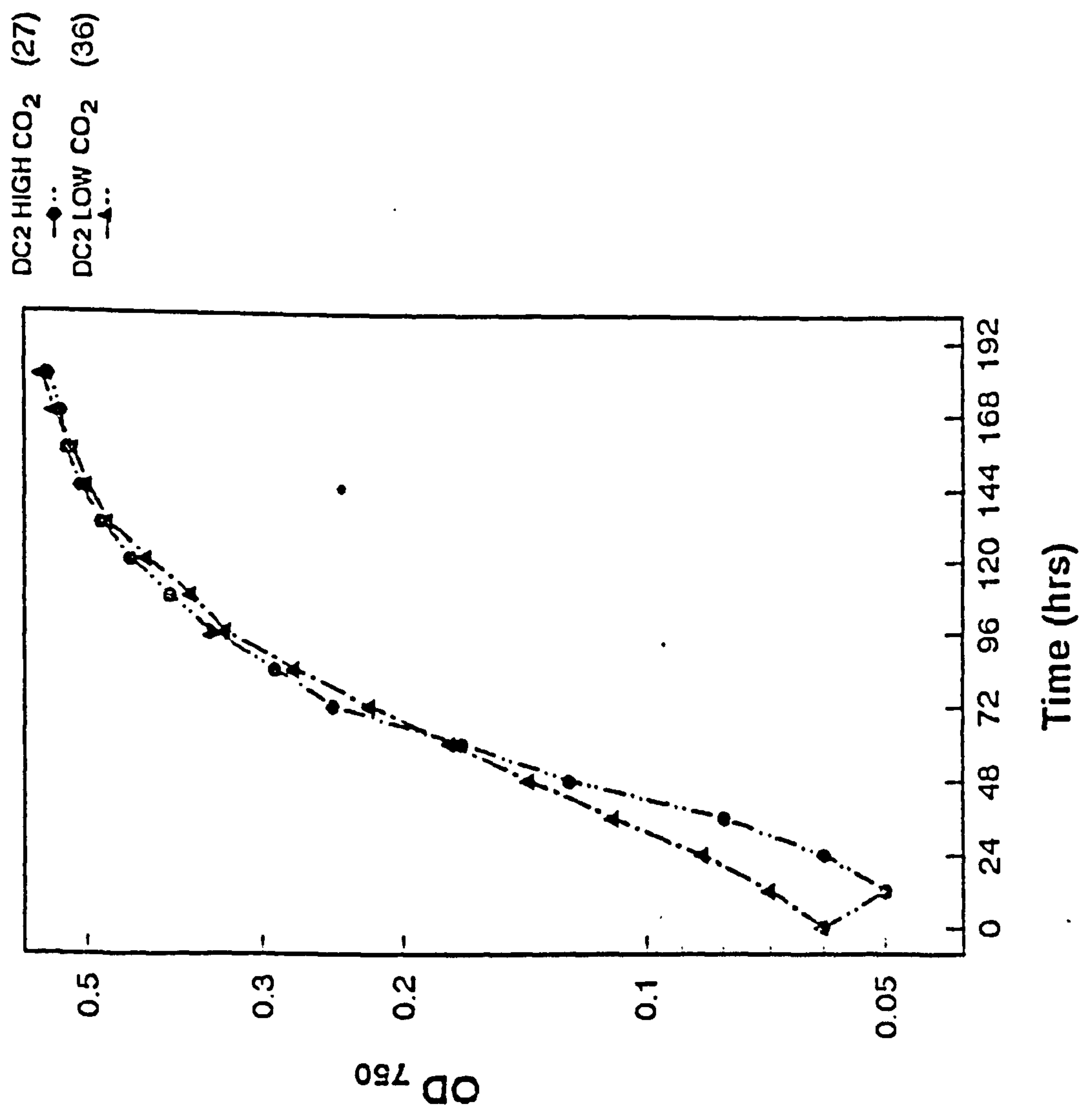


Figure 3.3 : Growth of Synechococcus DC2  
under high and low CO<sub>2</sub> regimes  
Figures in brackets represent doubling times of the organism





condition is probably related to some sort of control mechanism/environmental stimulus, such as cell division, rather than as an energy supply for the cells, a similar conclusion to that drawn by Anderson and McIntosh (1991). Photoheterotrophic growth in *Synechocystis* PCC6803 was also analysed on a variety of sugars and amino acids (fructose, maltose, galactose, pyruvate and malate) however no growth was detected using any of these substances.

### 3.3.2 RuBisCO activities of unicellular cyanobacteria grown under different carbon regimes

Table 3.1 summarises the results of the RuBisCO assay (see section 2.12. for methodology). In this study *in vitro* RuBisCO activities are much higher than the rates calculated by analysing the acid-stable counts in Ci uptake experiments, and Mayo et al. (1989), using *Synechococcus leopoliensis* UTEX625, have reported that this is consistent with the RUBP regeneration rate setting the maximum rate of photosynthesis.

In the majority of the species studied, the activity of RuBisCO appeared unaltered by the CO<sub>2</sub>-regime the strain was grown under. *A. quadruplicatum* BG1 showed a 2-fold increase in RuBisCO activity under high CO<sub>2</sub> conditions, whilst in two *Synechococcus* strains of the *Anacystis nidulans* type, a nearly 4-fold increase was observed under high CO<sub>2</sub> conditions. Working with different strains of cyanobacteria, Yokota and Calvin (1985) and Mayo et al. (1989) also found that RuBisCO activities were higher in high CO<sub>2</sub>-grown cells in batch culture and suggested that these

Table 3.1 : RuBisCO activity in batch cultures of cyanobacteria grown under different carbon regimes

Organism	RuBisCO activity (nmol CO <sub>2</sub> fixed mg dry wt <sup>-1</sup> min <sup>-1</sup> )		
	High CO <sub>2</sub> -grown	Low CO <sub>2</sub> -grown	Photoheterotrophically grown Low light      High light
<u>Synechococcus</u> PCC7942	35	10	
<u>Synechococcus</u> PCC6301	30	8	
<u>Synechocystis</u> PCC6803	9	12	1.63      2.14
<u>Synechocystis</u> PCC6308	24	22	
<u>Synechocystis</u> PCC6714	11	14	
<u>A. quadruplicatum</u> PR6	13	9	
<u>A. quadruplicatum</u> BG1	16	9	
<u>Synechococcus</u> WH7803	2	4	
<u>Synechococcus</u> WH8110	1.6	0.94	
<u>Synechococcus</u> WH8018	1.44	1.73	

differences seen may reflect a conservation of protein resources in low CO<sub>2</sub>-grown cells. An alternative explanation may be that there is an increase in the number of genome copies per cell, and the high enzymic activity observed in high as opposed to low CO<sub>2</sub>-grown cells may be a consequence of the increase in the number of RuBisCO genes per cell, as seen when *A. nidulans* is grown at fast growth rates (Mann and Carr, 1974).

The oceanic *Synechococcus* species (WH 7803, WH8018 and WH8110) all had reduced levels of RuBisCO activity compared with the freshwater species. As these oceanic species have considerably slower growth rates, this low enzymic activity may reflect a reduced CO<sub>2</sub>-fixation requirement per unit time. This suggestion would also explain the results obtained in photoheterotrophically grown *Synechocystis* PCC6803, where RuBisCO activity was at the same low levels as seen in the oceanic *Synechococcus*. sp. Although these photoheterotrophically grown cells also had much slower growth rates than seen in autotrophically grown cells, the low levels of RuBisCO activity seen most probably represent residual RuBisCO activities, the cell turning over the majority of its RuBisCO when autotrophy is no longer the major metabolic pathway used by the cell, although the rate of protein turnover is to a large extent relatively unknown in cyanobacteria.

The fact that these photoheterotrophically-grown cells can also still transport Ci (see Fig 3.4), although again at much lower rates than low CO<sub>2</sub>-grown cells may be part of an adaptive mechanism, enabling the cell to take immediate advantage of any



sudden influx of  $\text{CO}_2$  (or Ci) in the external environment.

### 3.3.3 Inorganic carbon uptake in unicellular cyanobacteria

#### 3.3.3.1 Silicone oil centrifugation

This method was the most widely used method to study Ci uptake and concentration in cyanobacteria and other species in the early-mid 1980's and was the method first used to examine the Ci uptake process in this study. An adaptation of the method of Miller and Coleman (1980) was used. These authors used 50  $\mu\text{l}$  of cell suspension layered on top of the silicone oil layer, and it was found in this study that this amount of cells gave too little pelleted material to enable accurate analysis of the Ci uptake process (data not shown), and so 180  $\mu\text{l}$  of cells were used.

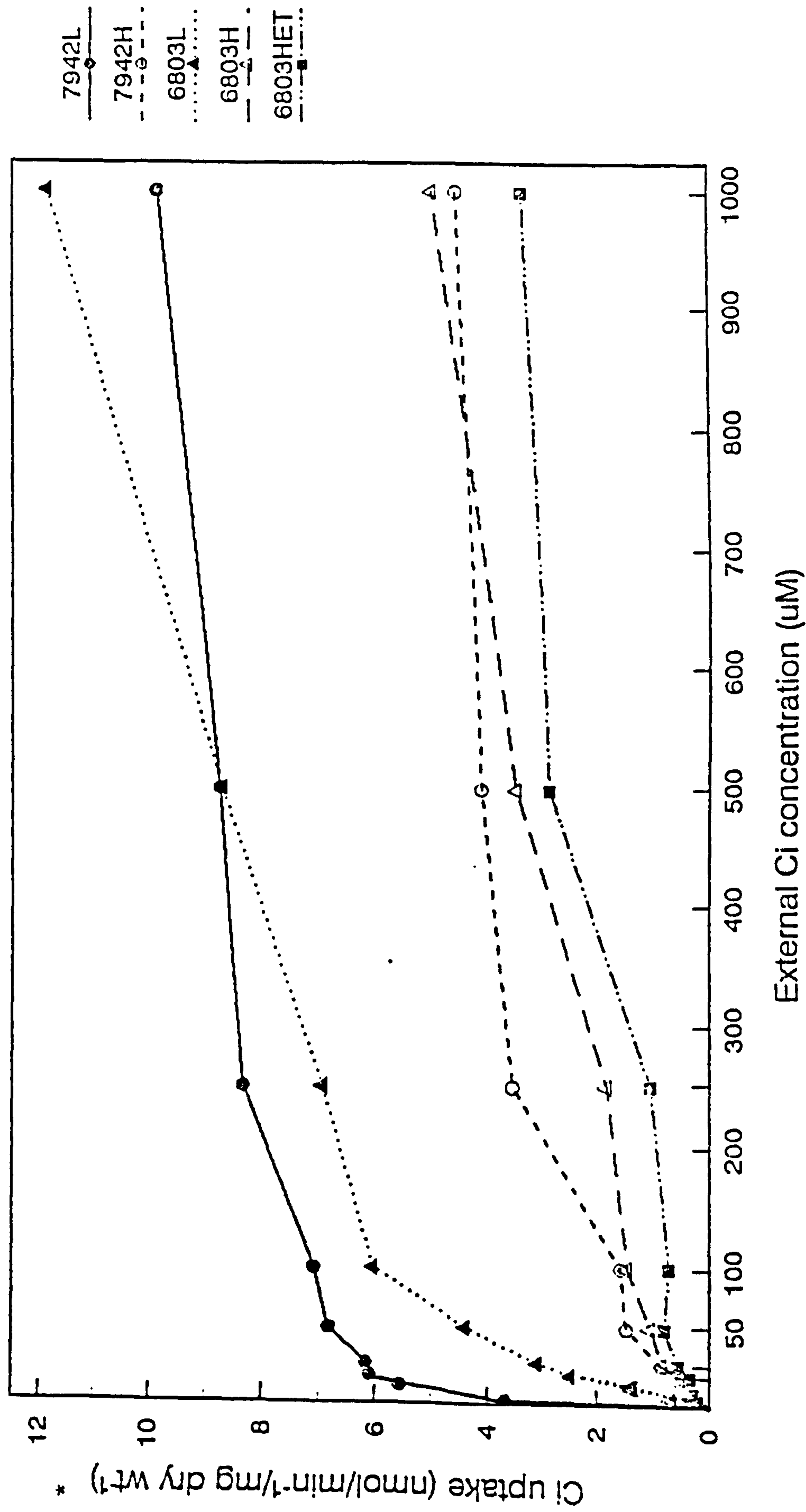
Figure 3.4 shows the rate of Ci uptake, under different growth conditions, into both *Synechocystis* PCC6803 and *Synechococcus* PCC7942 over a range of external Ci concentrations. The results were standardised for mg dry wt protein by assuming a 70% cell recovery after a 10 second spin in the microfuge centrifuge.

Incubations were performed for 30 seconds, since it was found that this incubation time enabled clear distinction between the internal Ci pool, which was usually between 70 - 85 % of the total Ci taken up by the cell (low  $\text{CO}_2$ -grown) and the Ci fixed into photosynthetic products (see section 3.3.3.2 and 3.3.3.3).

It can be seen that in both *Synechocystis* PCC6803 and *Synechococcus* PCC7942 the rate of Ci uptake is far greater in cells grown under low  $\text{CO}_2$  conditions, especially at the lower

Figure 3.4 : Rate of DIC uptake into *Synechocystis* PCC6803 and *Synechococcus* PCC7942 grown under different carbon regimes; assessment by silicone oil centrifugation

Incubations were carried out at 30°C, pH7.4 for 30 s : \* Ci uptake normalised for dry weight assuming 70% pelleting of cells  
7942L+7942H, *Synechococcus* PCC7942 grown under low and high CO<sub>2</sub>  
6803L,6803H+6803HET, *Synechocystis* PCC6803 grown under low and high CO<sub>2</sub> and heterotrophic conditions



bicarbonate concentrations used. In *Synechococcus* PCC7942 in particular, the difference in Ci uptake rates between low and high CO<sub>2</sub>-grown cells at external Ci concentrations below 50 µM was 10 - 15 fold. At the highest external DIC concentrations, the uptake rates start to level off, suggesting that the uptake system and the internal pools of Ci are becoming saturated. This is consistent with the work of other authors who have shown that there is a saturable, active Ci uptake and concentrating mechanism in low CO<sub>2</sub>-grown cyanobacteria, green algae and a limited range of other species (see review by Badger, 1987). Rates of Ci uptake in photoheterotrophically-grown *Synechocystis* PCC6803 were lower than those seen in high CO<sub>2</sub>-grown cells. This is as we would expect, because although the rate of Ci uptake in high CO<sub>2</sub>-grown cells is much lower than that seen in low CO<sub>2</sub>-grown cells, particularly at low external Ci concentrations, it has been shown that high CO<sub>2</sub>-grown cyanobacteria possess an active, low affinity, CO<sub>2</sub>-uptake mechanism (see reviews by Badger, 1987 and Miller, 1990). However, because the photoheterotrophically-grown cells have a different mode of metabolism, it is unlikely that the Ci uptake into these cells is active, unless the low affinity system seen in high CO<sub>2</sub>-grown cells is constitutive in this organism. It is more likely that the Ci that enters these cells does so by passive diffusion. If this is the case, the difference in uptake rates between the low and high CO<sub>2</sub>-grown cells and the photoheterotrophically grown cells represents the active uptake rates of the respective Ci uptake systems under these growth conditions.



In all experiments performed using the silicone oil centrifugation technique, each experiment was performed in triplicate, and in Figure 3.4 each point represents the average of these three readings. Although Figure 3.4 makes no distinction between the Ci incorporated into acid stable photosynthetic products and the acid labile Ci pool, this was performed by acidifying one half of each sample obtained. The reason why this chapter devotes so little time to the silicone oil centrifugation methodology is that the method was found to be inconsistent, and the data obtained in Figure 3.4 was the only reliable data obtained over three months work using the system. Although the work in Figure 3.4 has assumed a 70% pelleting of the cells to calculate dry weights, discrepancies of 30 - 40 % in the pelleting often occurred between parallel samples which had been treated in an identical fashion. It was sometimes possible to detect differences in the pelleting efficiency of identical samples visually by comparing pellet sizes and the amount of cells which remained behind at the interface of the incubation mix/silicone oil interface. To try and eliminate this discrepancy it was decided to try and quantify each sample by assessing the chlorophyll content in a fluorimeter. However, it was found that this method required too much of the sample, leaving too little to carry out analysis of the Ci uptake characteristics. In an attempt to improve upon the current methodology, it was decided to label each cell internally, so that a direct analysis of the Ci concentrating system could be made against a known amount of internal marker.

### 3.3.3.2 Silicone oil centrifugation using dual labelled cells

In determining the compound to be used as an internal marker to quantify Ci uptake by the silicone oil centrifugation method, the chosen compound had to be readily taken up by the strain being analysed. It was decided to use  $^3\text{H}$ -leucine as a marker, because it was known to have an active transport mechanism in *Synechocystis* PCC6803 (see Labarre et al., 1987), the chosen strain for this particular study.

It was decided to use the tritium, because it was possible to set up the scintillation counter windows so that the disintegrations from both of these radioisotopes could be distinguished. The windows in the scintillation counter were set up so that 100% of the  $^3\text{H}$  counts were read in window one, whilst the  $^{14}\text{C}$  counts, which were read in window two, had a cross channel compensation factor added in the final calculations on the spread sheet (roughly  $\times 1.5$ ) to enable accurate counting of the Ci taken up. In addition it was found using the internal standards in the scintillation counter that the efficiency of counting for these two radioisotopes was 67% for  $^3\text{H}$  and 98% for  $^{14}\text{C}$  respectively and this was also compensated for in the final calculations.

Although workers such as Labarre et al. (1987) have shown that leucine is taken up by *Synechocystis* PCC6803, it had to be established that the tritiated form of leucine was taken up under all of the growth conditions employed. Incubation of cells grown under high and low  $\text{CO}_2$  and photoheterotrophic conditions

with a dilution series of radiolabelled:unlabelled leucine from  $10^{-3}$ - $10^{-4}$ M revealed that leucine was taken up from the external medium at all of the leucine concentrations used, and it was decided to carry out further work using a leucine concentration of  $10^{-4}$ M, as this concentration of leucine enabled sufficient radioisotope incorporation into the cells to quantify leucine uptake with other cellular parameters (see Table 3.2) and yet leave sufficient leucine in the medium throughout the growth cycle (data not shown).

In an attempt to quantify the leucine taken up by *Synechocystis* PCC6803 with other cellular parameters, leucine uptake was measured throughout the growth cycle and correlated with chlorophyll content and dry weight (see Table 3.2). It can be seen that there is a good correlation in the leucine uptake per mg dry weight for the particular growth phase the cells are in, and during mid-logarithmic phase, where the cells are harvested for Ci uptake analysis, this corresponds to around 25.0 nmole leucine per mg dry weight. As growth enters the late logarithmic/stationary phase this figure drops to around 20.0 nmole per mg dry weight, which would suggest that the leucine is being depleted from the external medium. However, analysis of the leucine left in the growth medium showed this was not the case, and it is likely that the slowing down in the rate of metabolism of the cells upon entering late logarithmic/stationary phase which will result in a decrease in the activity/amount of certain enzymes, and hence a corresponding drop in leucine incorporation, is the reason. The



Table 3.2 : Incorporation of <sup>3</sup>H-leucine into Synechocystis PCC6803; correlation with dry weight and chlorophyll concentration

Growth condition	Exponential stage of growth cycle							
	early				mid			
	OD750	chlorophyll content <sub>1</sub>	dry wt <sub>2</sub>	OD750	chlorophyll content	dry wt	OD750	chlorophyll content
								dry wt
Low CO <sub>2</sub>	0.175	58	29	0.398	75	24	0.823	87
High CO <sub>2</sub>	0.214	72	34	0.468	64	26	0.907	81
Heterotrophic low light	0.261	46	36	0.426	67	24	0.714	94
Heterotrophic high light	0.202	64	39	0.477	82	26	0.774	67

1 Figures for chlorophyll are expressed as nmole leucine per ug chlorophyll

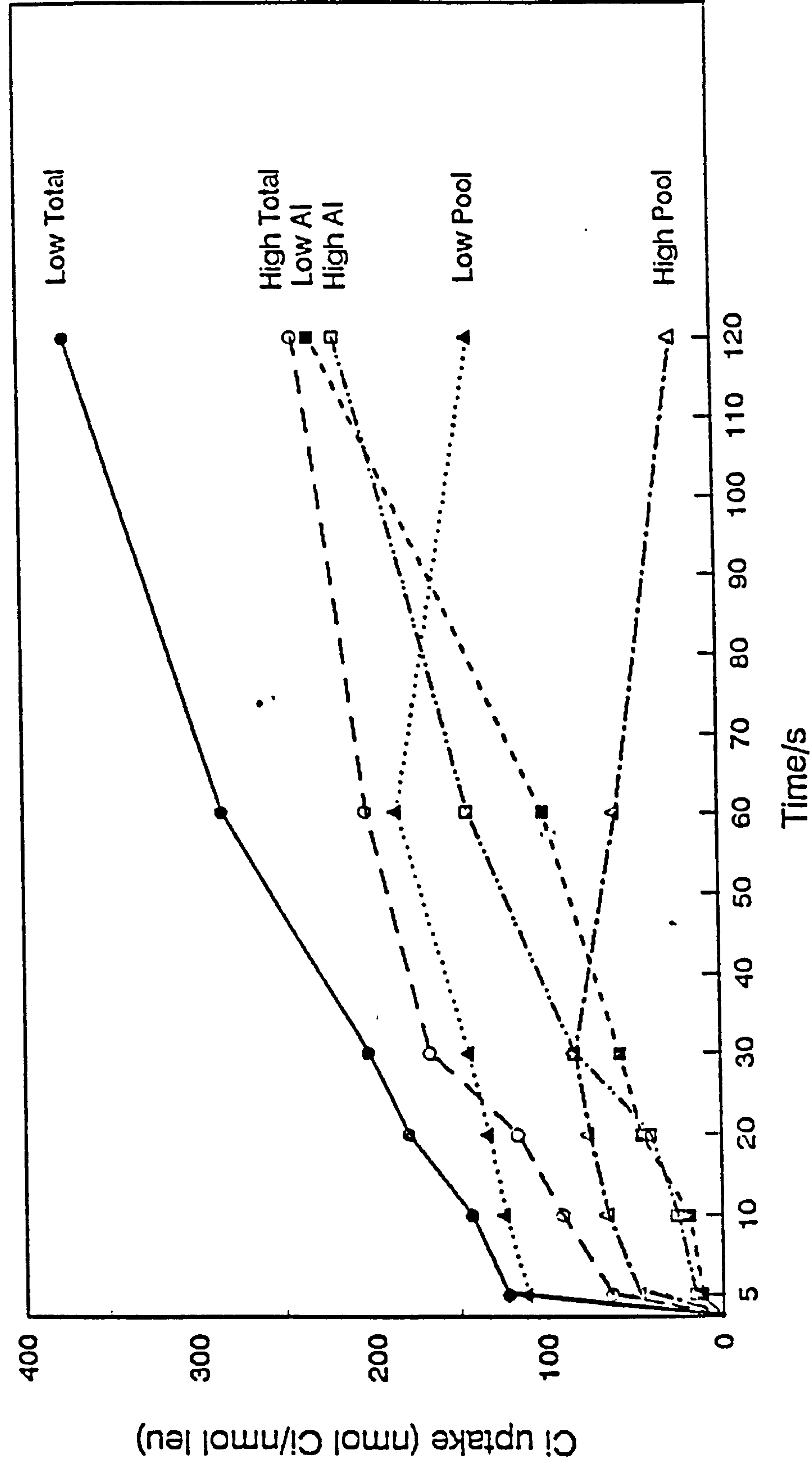
2 Figures for dry weights are expressed as nmole leucine per mg dry weight

correlation with chlorophyll content did not prove as strong, and in particular was weak amongst the photoheterotrophically grown cells. This is understandable, because as an amino acid leucine will probably become incorporated into protein, and so is far more likely to show good correlation with dry weight than chlorophyll content.

Figure 3.5 shows the time course of Ci uptake and accumulation at an external Ci concentration of 100  $\mu\text{M}$  at pH 7.4 in high and low  $\text{CO}_2$ -grown *Synechocystis* PCC6803 labelled with tritiated leucine. There is a rapid uptake of Ci over the first five seconds in both high and low  $\text{CO}_2$ -grown cells, with the majority of the Ci entering the internal Ci pool. Uptake of Ci in low  $\text{CO}_2$ -grown cells was far greater than in high  $\text{CO}_2$ -grown cells. Whereas uptake into the internal Ci pool in high  $\text{CO}_2$ -grown cells remained fairly constant until 30 seconds, when it started to decline due to the onset of photosynthesis, there was a steady increase of Ci into the internal Ci pool in low  $\text{CO}_2$ -grown cells, up to 60 seconds. In both cell types there was a lag in  $\text{CO}_2$  fixation (as expressed by acid-insoluble Ci) whilst Ci accumulated in the cells, and once the internal pool sizes had built up to a sufficient size, there was a rapid onset of  $\text{CO}_2$  fixation. This led to a rapid depletion in the internal Ci pool in high  $\text{CO}_2$ -grown cells, and by 120 seconds there was only a small internal Ci pool, with virtually all of the Ci taken up by the cell being immediately fixed into photosynthetic products, which suggests that these cells only have a limited ability to maintain an internal Ci pool. In low  $\text{CO}_2$ -grown cells on the

Figure 3.5 : Time course of Ci uptake and accumulation in high and low CO<sub>2</sub> grown  
*Synechocystis* PCC6803 labelled with tritiated leucine

External DIC concentration 100  $\mu$ M, 30°C, pH7.4 : Low and high refer to low and high CO<sub>2</sub> grown  
*Synechocystis* PCC6803  
 Total, total Ci uptake ; AI, acid insoluble Ci (photosynthetic products) ; Pool, intracellular free Ci pool





contrary, although the Ci pool size declined following the onset of photosynthesis, the pool was maintained at relatively high levels throughout the time course, and even after 120 seconds there was an appreciable pool present. This demonstrates that these low CO<sub>2</sub>-grown cells of *Synechocystis* PCC6803 possess a mechanism capable of establishing and maintaining an internal Ci pool. This internal Ci pool has been suggested by many workers to raise the intracellular CO<sub>2</sub> concentration around RuBisCO, enabling photosynthesis to occur in conditions when the external CO<sub>2</sub> concentration would make direct fixation by RuBisCO inconsequential (see review by Badger, 1987). This experiment has shown that a similar mechanism is present in *Synechocystis* PCC6803 as seen in various other species for establishing and maintaining an internal Ci pool, and has shown the effectiveness of radiolabelling the cells prior to silicone oil centrifugation. With reference to Table 3.2 the conversion to nmoles Ci per mg dry weight is easily made, and this shows good correlation with the work performed using vacuum filtration. Although this methodology improves upon the silicone oil centrifugation method, enabling an accurate quantification of the amount of material centrifuged through the silicone oil, it did not make the methodology any quicker, and the fact that tritiated leucine was being used in the growth medium made the experiments expensive and potentially dangerous. An alternative method was sought which gave consistently reproducible results and allowed the rapid analysis of large numbers of samples, and the vacuum filtration method (see section 2.10.1) was found to

fulfill these requirements.

#### 3.3.3.3 Vacuum filtration

This method, in which cells are separated from their incubating medium by filtration through a membrane or filter attached to a vacuum filtration apparatus has been the most widely used method for studies of bacterial transport (see Miller 1990), but with the exception of Holthuijzen et al. (1987) has not been widely used to study Ci transport. The main reservation with this method in the study of Ci uptake has been the leakage of accumulated Ci during filtration (Miller, 1990). As will be shown later in this study, this did not affect the results obtained, which were comparable with the silicone oil centrifugation method.

Before any Ci uptake experiments were performed, the "quenching" effect of various filters and pretreatments on the efficiency of counting was determined. As can be seen from Table 3.3 the effects of adding 200 ul perchloric and phosphoric acid at various molarities (which were used to acidify the sample to drive off the Ci in the internal pool) as well as the effects of both Whatman GFF and 0.2 and 0.45 um nitrocellulose filters on the efficiency of  $^{14}\text{C}$  counting in the scintillation counter were negligible.

Other experiments were also performed to establish the filters which gave the best cell retainment and again there was no appreciable difference between Whatman GFF filters and nitrocellulose filters (data not shown), and so it was decided

Table 3.3 : Quench data for vacuum filtration

Figures shown are the counts per minute (cpm) obtained from 10 ul of a 10 uM sample of  $\text{NaH}^{14}\text{CO}_3$ .

Treatment	Scintillation counter channel			
	1 cpm	2 cpm	3 cpm	4 cpm
Standard	4483.42	17904.12	11322.56	24746.85
	4543.24	18069.19	11667.77	24894.42
Whatman GF/F	4224.33	18268.94	11525.80	24769.99
	4449.63	18237.91	11410.98	25002.50
nitrocellulose 0.2 um	4633.14	18555.83	11847.51	25651.84
nitrocellulose 0.45um	4698.45	18326.42	11490.29	25491.99
Perchloric acid 0.1M	4914.52	17045.05	9844.73	24494.32
Perchloric acid 0.2M	5579.59	16592.68	8877.48	25054.24
Perchloric acid 0.5M	6492.32	15010.37	6389.81	24721.64
Perchloric acid 1.0M	7210.54	12826.59	4085.71	23668.13
Phosphoric acid 0.1M	4652.83	17905.00	10789.19	25000.65
Phosphoric acid 0.2M	4624.61	17087.71	10163.21	24199.89
Phosphoric acid 0.5M	5480.90	16674.16	8615.30	24972.04
Phosphoric acid 1.0M	5085.86	15646.96	7898.26	23452.83



to carry out all experiments using GFF filters. Initially 0.1 M NaOH was added to the reaction mix to terminate the reaction, prior to filtering, however it was found that this occasionally led to cell lysis, and so was abandoned as it was found by comparison that there was no appreciable difference between Ci uptake taken up in cells that had been treated with 0.1 M NaOH as opposed to those that were placed directly onto GFF filters (data not shown).

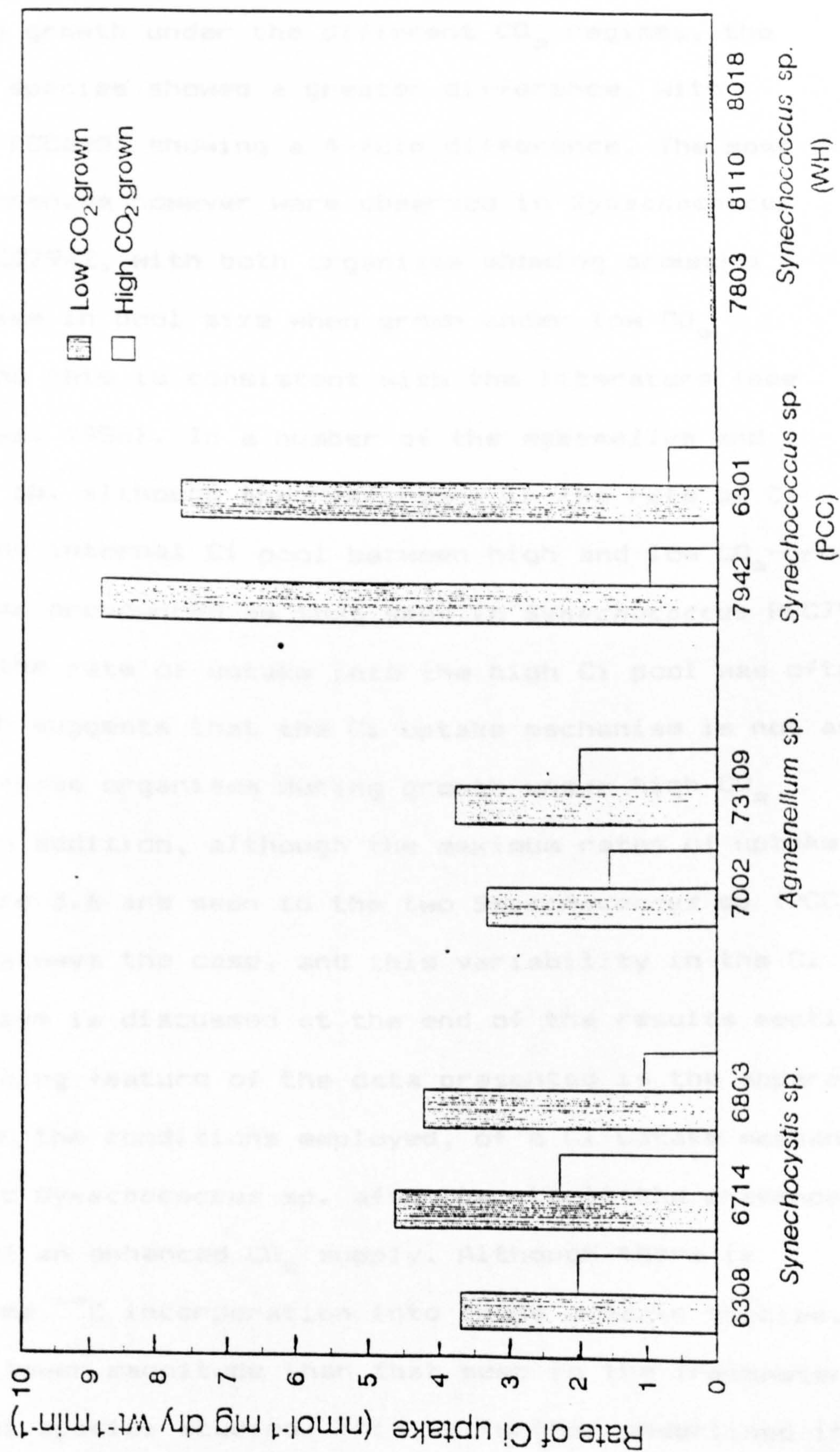
Figure 3.6 shows the rate of Ci uptake at an external Ci concentration of 100  $\mu$ M, pH 7.4, into the intracellular Ci pool in a variety of unicellular cyanobacteria after growth under high and low CO<sub>2</sub> regimes. In considering the data on Ci uptake it is important to clarify the terms for the areas these organisms are capable of growth. Freshwater is self explanatory; marine has often been used to describe organisms which were isolated from any part of the marine environment, including intertidal areas. Consequently such organisms may exhibit facultative dependence for 3% NaCl, an example being the *Agmenellum* species employed in this study (see Rippka et al., 1979). The term oceanic is used in this study to describe organisms isolated from open waters and which have an absolute dependence for 3% salt. A characteristic feature of all of the oceanic species employed in this study was the presence of phycoerythrin as an accessory pigment.

All of the organisms capable of growth in freshwater showed varying degrees of ability to transport and maintain an internal pool of Ci; all had a larger internal Ci pool when grown under



Figure 3.6 : Rate of  $\text{Ci}$  uptake into the internal  $\text{Ci}$  pool of several unicellular cyanobacteria following growth under high or low  $\text{CO}_2$

External DIC concentration 100  $\mu\text{M}$ , 30°C, pH7.4.





low CO<sub>2</sub> conditions. The *Agmenellum* species showed a relatively modest alteration in the rate of Ci uptake into the internal pool following growth under the different CO<sub>2</sub> regimes, the *Synechocystis* species showed a greater difference, with *Synechocystis* PCC6803 showing a 4-fold difference. The most dramatic differences however were observed in *Synechococcus* PCC6301 and PCC7942, with both organisms showing around a 10-fold increase in pool size when grown under low CO<sub>2</sub> conditions, and this is consistent with the literature (see Omata and Ogawa, 1986). In a number of the *Agmenellum* and *Synechocystis* sp. although the difference in the rate of Ci uptake into the internal Ci pool between high and low CO<sub>2</sub>-grown cells is not as pronounced as that seen in *Synechococcus* PCC7942 and PCC6301, the rate of uptake into the high Ci pool was often greater, which suggests that the Ci uptake mechanism is not as repressed in these organisms during growth under high CO<sub>2</sub> conditions. In addition, although the maximum rates of uptake shown in Figure 3.6 are seen in the two *Synechococcus* sp (PCC) this was not always the case, and this variability in the Ci uptake mechanism is discussed at the end of the results section. The most striking feature of the data presented is the apparent absence, under the conditions employed, of a Ci uptake mechanism in the oceanic *Synechococcus* sp. after growth in the presence and absence of an enhanced CO<sub>2</sub> supply. Although there is definitely some <sup>14</sup>C incorporation into these oceanic species, it is of a much lower magnitude than that seen in the freshwater cyanobacterial species studied. This is further underlined in

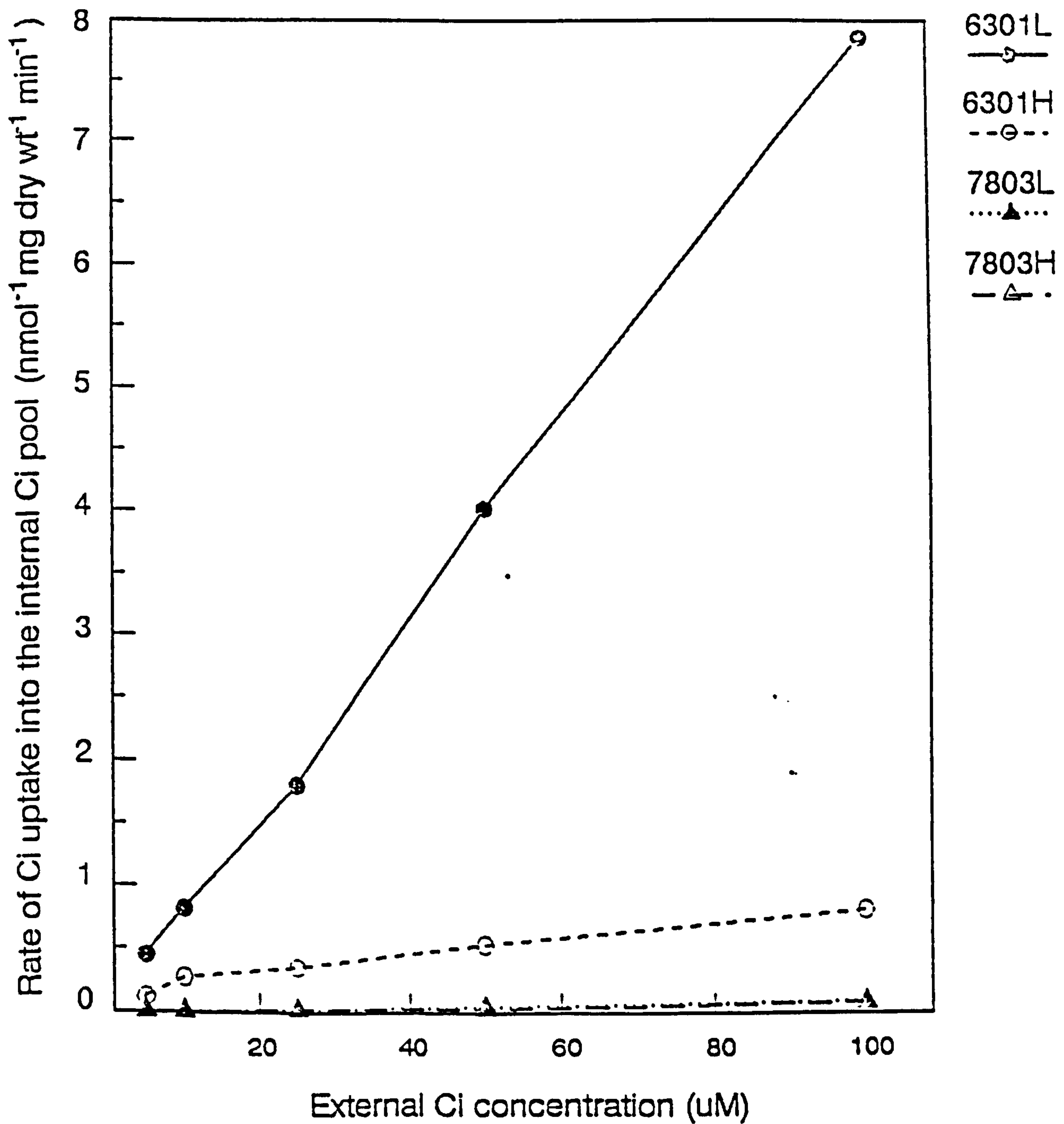


Figure 3.7, which shows the accumulation of Ci into the internal Ci pool in both *Synechococcus* PCC6301 and *Synechococcus* WH7803 over a range of external Ci concentrations. *Synechococcus* PCC6301 shows the typical response of an organism possessing a Ci uptake mechanism, with much greater quantities of Ci being transported into the pool in low CO<sub>2</sub>-grown cells, with appreciable amounts of Ci being transported into high CO<sub>2</sub>-grown cells only at the higher external Ci concentrations used. In contrast, there was no appreciable difference between the amounts of Ci transported into *Synechococcus* WH7803, after growth in the presence or absence of an enhanced CO<sub>2</sub>, at any of the external Ci concentrations used. In addition, the amounts of Ci transported were far lower than those seen in either low or high CO<sub>2</sub>-grown *Synechococcus* PCC6301, which would seem to confirm the absence of a Ci uptake mechanism in the oceanic species under the conditions employed.

It is noteworthy that Glover and Morris (1981) observed photorespiration in the oceanic species *Synechococcus* DC2 (WH7803), a phenomenon frequently suppressed in cyanobacteria due to their Ci concentrating ability (see Ogren, 1984). CO<sub>2</sub> concentration has however been reported in several eukaryotic marine microalgae, including *Oscillatoria woronichinii* (Burns and Beardall, 1987), although it is not clear whether these were true oceanic or marine species as defined above.

Restriction length polymorphism analysis has indicated a high degree of separation between the oceanic *Synechococcus* and freshwater *Synechococcus* sp. (Douglas and Carr, 1988), and the

Figure 3.7 : Accumulation of Ci into the internal Ci pool in *Synechococcus* 6301 and 7803 in response to the external Ci concentration



6301L + 6301H, *Synechococcus* PCC6301 grown under low and high CO<sub>2</sub>  
 7803L + 7803H, *Synechococcus* WH7803 grown under low and high CO<sub>2</sub>

absence of a  $\text{Ci}$  uptake mechanism under the conditions employed further removes the oceanic *Synechococcus* sp. from the freshwater unicellular *Synechococcus* sp. studied.

The reason behind this lack of a  $\text{Ci}$  uptake mechanism may lie in the nature of the environment from which the organisms were isolated. Open seawater has a bicarbonate concentration of around 1.8 mM (Round, 1981), much higher than anything but the most alkaline freshwater system (where in any case the majority of the DIC will be in the form of carbonate). Both Mayo et al. (1986 and 1989) and Badger and Gallacher, 1987) have reported that above an external DIC concentration of 1.5–2.0 mM *Synechococcus* PCC6301 is repressed in terms of  $\text{Ci}$  uptake. For the oceanic *Synechococcus* sp., growth in an environment with consistently high bicarbonate concentrations coupled with the smaller size (and hence large surface/volume ratio) and relatively slow growth rates of these oceanic *Synechococcus* species (see Figure 3.3) may mean that these organisms never become rate limited with respect to available bicarbonate supply. If this is the case, the selective pressure to develop and maintain the energetically expensive  $\text{Ci}$  concentrating process (see Raven and Lucas, 1985) would not exist. Obviously this hypothesis will have to be validated, and once the various components of the  $\text{Ci}$  uptake mechanism have been identified it should prove relatively simple to raise antibodies to the components and probe natural populations of these oceanic *Synechococcus* sp. to see if they possess such a system. This obviously assumes that if such a system exists it will be of a



mechanistically similar nature, constructed of similar polypeptides. With what is already known of the oceanic *Synechococcus* species in relation to the freshwater *Synechococcus* species (see Douglas and Carr, 1988) this may not be the case).

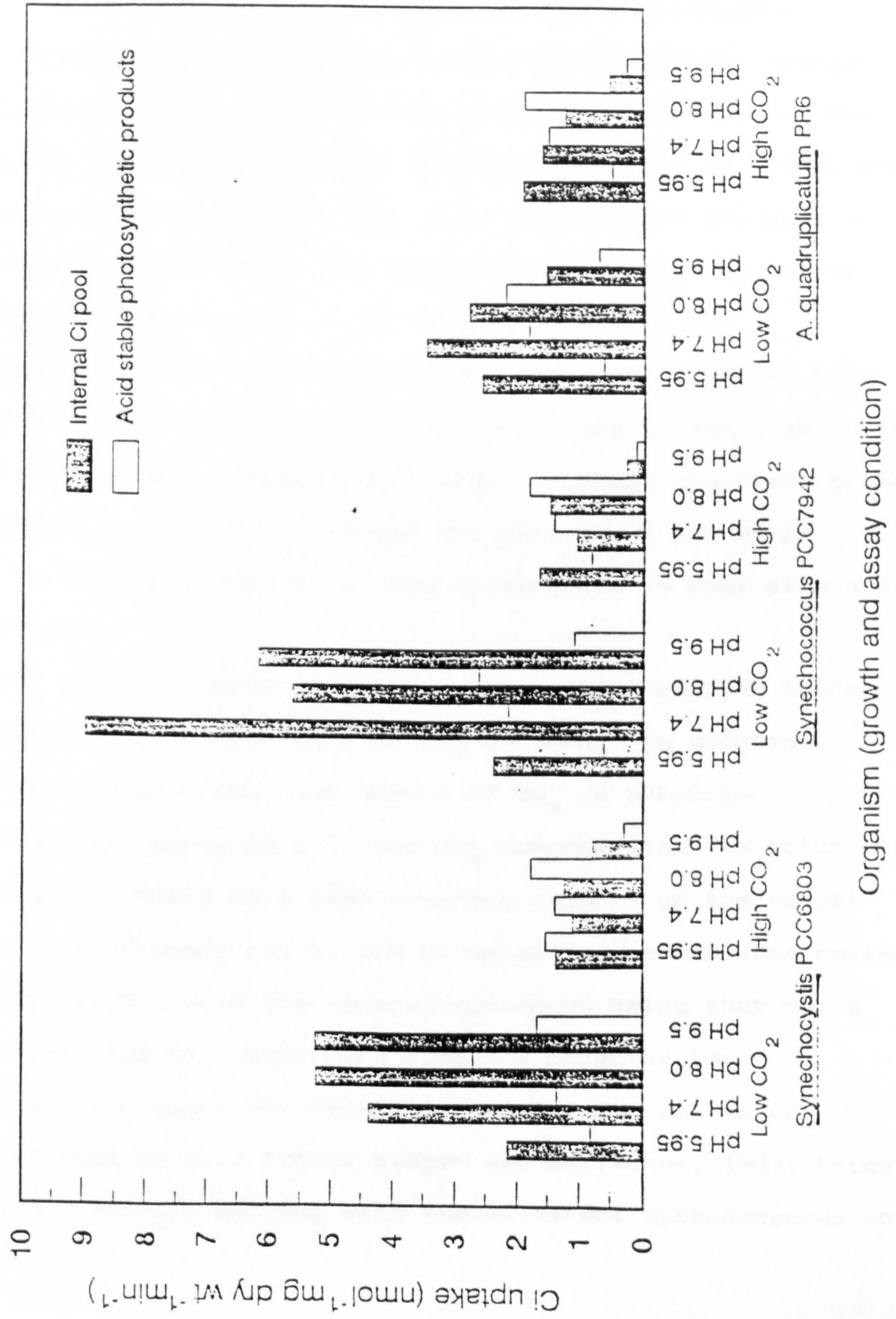
#### 3.3.3.4 Effect of changes in the external pH on the $C_i$ uptake mechanism

Figure 3.8 shows the effect of varying the external pH in the reaction mix, by the use of biological buffers, in one of each of the three representative groups of unicellular cyanobacteria used in this study (the oceanic species were also tested, but again did not show any signs of a  $C_i$  uptake and concentration mechanism and so have been omitted).

In all of the organisms studied, the optimum pH for photosynthesis (as determined by acid insoluble counts) was between pH 7.4 and 8.0. In conditions that were acidic (pH 5.95) or extremely alkaline (9.5) photosynthesis was reduced. The only exception to this was *Synechocystis* PCC6803, in which  $C_i$  fixed at pH 5.95 in high  $CO_2$ -grown cells, and pH 9.5 in low  $CO_2$ -grown cells was the same as at pH 7.4 and 8.0. Coleman and Colman (1981) and Shelp and Canvin (1984) have reported that this inhibition of photosynthesis at acidic and strongly alkaline conditions is due to the inability of the organism to maintain its internal pH. Transport of  $C_i$  into the internal  $C_i$  pool in low  $CO_2$ -grown cells was more variable amongst the species studied. In both the *Synechococcus* and *Synechocystis* sp. uptake



Figure 3.8 : Effect of external pH on rate of  $\text{Ci}$  uptake in several unicellular cyanobacteria grown under different carbon regimes





of Ci into the Ci pool was significantly reduced in acidic conditions, but remained relatively unchanged between pH 7.4 and 9.5. The *Agmenellum* sp. did not show any reduction in the size of the Ci pool in acidic conditions, however in extremely alkaline conditions, the pool size was reduced by 50%. Amongst the organisms grown under high CO<sub>2</sub> conditions, the size of the internal Ci pool was relatively constant from pH 5.95 - 8.0, but was much smaller at pH 9.5. This is in contrast to the work of Coleman and Colman (1981), who found that the capacity for Ci transport was not reduced at pH values inhibitory for photosynthesis. If a comparison between the pool sizes of high and low CO<sub>2</sub>-grown cells over the pH range used is made, above pH 7.4 the pool size is always much larger in those organisms grown under low CO<sub>2</sub>. At pH 5.95, whilst the pool sizes are still larger in low CO<sub>2</sub>-grown cells, the differences in pool size are much smaller.

If Figure 1.5 is studied it can be seen that at pH 5.95 there are roughly equal proportions of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> in solution, however as the pH rises, the amount of CO<sub>2</sub> in solution diminishes, and above pH 8.2, the CO<sub>2</sub> concentration in solution is negligible. There have been numerous reports on the actual species of Ci transported by the Ci uptake mechanism (see review by Miller, 1990), with the general consensus being that cells grown under high CO<sub>2</sub> conditions lose the capacity for bicarbonate transport but retain a capacity for active CO<sub>2</sub> transport (Abe et al., 1987a; Badger and Gallacher, 1987; Price and Badger, 1989a). Working with two different *Synechococcus* sp.



Badger and Andrews (1982) and Badger and Gallacher (1987) found that  $\text{CO}_2$  was more readily taken up when supplied at an equivalent concentration to  $\text{HCO}_3^-$ . This work ties in with the results obtained here. Some workers have found a five-fold reduction in the affinity for  $\text{CO}_2$  transport in high as opposed to low  $\text{CO}_2$ -grown cells (Badger and Gallacher, 1987), however Shelp and Calvin (1984) working with *A.nidulans* found little difference in the affinity for Ci in low and high  $\text{CO}_2$ -grown cells at low pH. In this study, at pH 5.95, where there are roughly equivalent concentrations of  $\text{CO}_2$  and  $\text{HCO}_3^-$  in solution, the uptake of Ci into the internal pool is equivalent in cells grown under high and low  $\text{CO}_2$ , suggesting that in the high  $\text{CO}_2$ -grown cells in particular  $\text{CO}_2$  is being transported into the cells. The slightly higher uptake values seen in low  $\text{CO}_2$ -grown cells may reflect their ability to transport bicarbonate as well as  $\text{CO}_2$ . The increased Ci uptake rates in low  $\text{CO}_2$ -grown cells as the pH is increased, is consistent with the work of others (see review by Miller, 1990) who suggest that the differences in the Ci uptake and concentrating ability at these higher pH's are the result of the ability to these low  $\text{CO}_2$ -grown cells to transport bicarbonate.

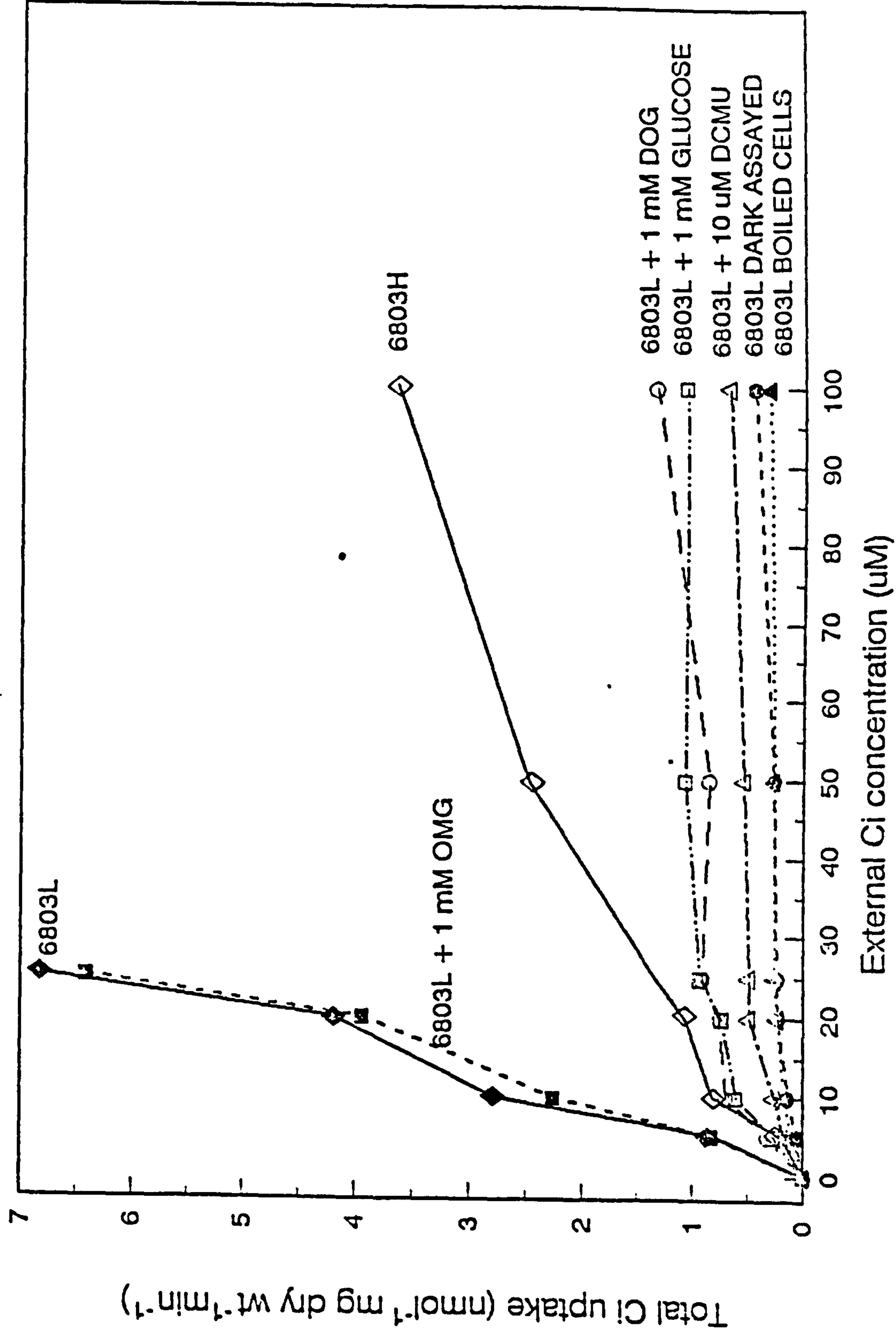
#### 3.3.3.5 Ci uptake under various growth and assay conditions in *Synechocystis* PCC6803

Figure 3.9 shows the effects on Ci uptake in *Synechocystis* PCC6803, over a range of external Ci concentrations (pH 7.4) following growth on a variety of compounds, as well as under

Figure 3.9 : Ci uptake under various growth and assay conditions  
in *Synechocystis* PCC6803

Assay time 30 s, pH 7.4, 30°C

L, Low CO<sub>2</sub>; H, High CO<sub>2</sub>; OMG, 3-O-methyl-D-glucose ; DOG, 2-deoxy-D-glucose



various assay conditions. If we consider the data obtained from the various assay conditions first, it can be seen that there is no appreciable uptake of  $\text{Ci}$  in low  $\text{CO}_2$ -grown *Synechocystis* PCC6803, following addition of 10  $\mu\text{M}$  DCMU into the reaction mix, in cells boiled prior to the assay or in cells assayed in the dark. These conditions were also used to study  $\text{Ci}$  uptake in the other unicellular cyanobacteria used in this study and the results were very similar, namely a substantial reduction in the ability to transport  $\text{Ci}$  (data not shown). This is consistent with the evidence to date regarding the  $\text{Ci}$  uptake system, which is known to be dependent on both PS1-driven cyclic electron flow and ATP synthesis (Ogawa et al., 1985). The rest of the data is concerned with  $\text{Ci}$  uptake following growth under certain conditions. In the instances where glucose, or glucose analogues have been added to low  $\text{CO}_2$ -grown *Synechocystis* PCC6803, this occurred 24 hours before the assay took place since it has been shown in *C. reinhardtii* that the process of adaptation to high  $\text{CO}_2$  conditions took 20 h (Marcus et al., 1982). Uptake of  $\text{Ci}$  was very rapid in low  $\text{CO}_2$ -grown *Synechocystis* PCC6803, and although not shown in Figure 3.9, at 100  $\mu\text{M}$  external  $\text{Ci}$  concentration, the rate of uptake was  $17 \text{ nmol}^{-1} \text{ mg dry wt}^{-1} \text{ min}^{-1}$ . This rate of  $\text{Ci}$  uptake was similar in low  $\text{CO}_2$ -grown cells to which had been added 1 mM of the glucose analogue 3-O-methyl-glucose (OMG), with rates being around 10% lower than in low  $\text{CO}_2$ -grown cells. In the remaining cultures,  $\text{Ci}$  uptake was greatly reduced. In high  $\text{CO}_2$ -grown cells, rates of uptake were above those seen following addition of glucose or the glucose analogue

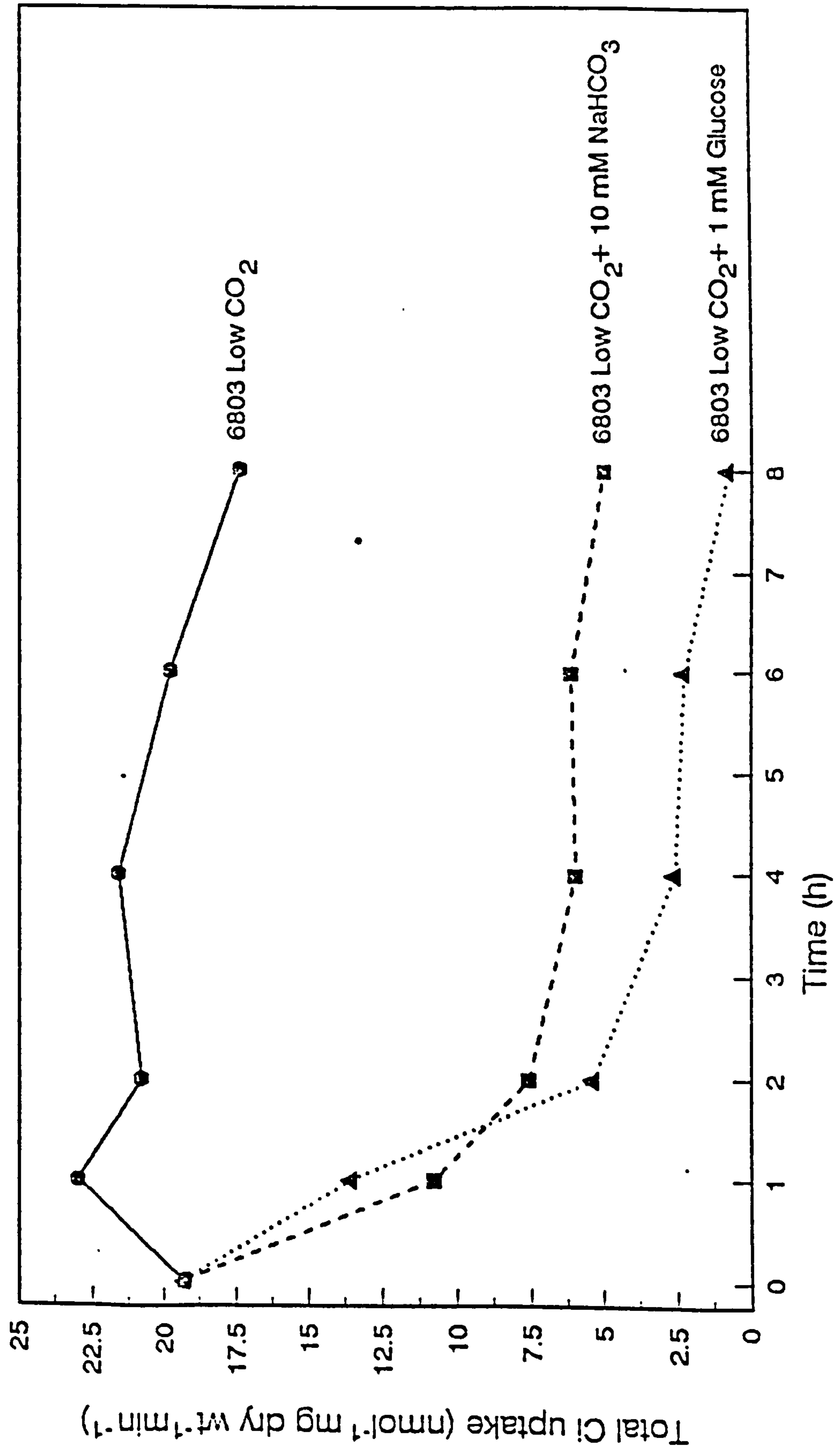


2-deoxy-D-glucose (DOG), but were much lower than the rates seen in low CO<sub>2</sub>-grown cells, consistent with the results so far shown. Surprisingly, the glucose analogue DOG had a very profound effect on the uptake rates in low CO<sub>2</sub>-grown cells, and its addition resulted in a severe inhibition in Ci uptake, similar to the effect of glucose. Beauclerk and Smith (1978) have reported that DOG is taken up by *Synechocystis* PCC6714, a close relative of *Synechocystis* PCC6803, and as this analogue had such a profound effect on Ci uptake in low CO<sub>2</sub>-grown cells of *Synechocystis* PCC6803, it obviously can enter the cell. The analogue OMG, although it did not cause a substantial reduction in the Ci uptake rate, is known to be taken up by *Synechocystis* PCC6803 (Flores and Schmetterer, 1986; Joset et al., 1988), so the lack of inhibition is not due to the fact that this substance cannot enter the cell. The fact that these two non-metabolizable analogues of glucose exert different effects on the Ci uptake mechanism, must be related to their structure. As both analogues can enter the cell, the inhibition of Ci uptake is unlikely to occur upon transport across the cell membrane by the glucose transport system. The only known fate of glucose (or the analogues) upon entering the cell is phosphorylation by ATP, catalysed by hexokinase, to form glucose-6-phosphate. It is hypothesized that the structural analogue DOG, like glucose is phosphorylated, and a build-up of glucose-6-phosphate (or DOG-6-phosphate) switches the Ci concentrating mechanism off. OMG, with its side chain addition, cannot enter the active site of hexokinase, and so remains

unphosphorylated. As no phosphorylated analogue builds up, the Ci uptake mechanism is not inhibited. Interestingly, a build-up of phosphoglycollate has been reported to be the signal for the switch-off of the Ci uptake mechanism in *A. variabilis* (Marcus et al., 1982) and it may be that build-up of any one of several different phosphorylated intermediates leads to the Ci uptake mechanism being switched off.

Following the observations in Figure 3.9 that addition of glucose (and DOG) 24 hours before assaying low CO<sub>2</sub>-grown cells for their Ci uptake ability caused inhibition of Ci uptake, it was decided to follow more closely the decay in the Ci uptake rates (see Figure 3.10). In low CO<sub>2</sub>-grown *Synechocystis* PCC6803 following addition of 1.0 mM glucose it can be seen that over the 8 hour time course the Ci uptake rate of low CO<sub>2</sub>-grown cells falls from around 20 to below 1.0 nm<sup>-1</sup> Ci/mg dry wt<sup>-1</sup>/min<sup>-1</sup>, with a 50% decay occurring in just over one hour. Addition of 10 mM NaHCO<sub>3</sub> also caused a rapid decay in the Ci uptake mechanism of low CO<sub>2</sub>-grown cells, after 8 hours Ci uptake rates were roughly 20% of those seen in low CO<sub>2</sub>-grown cells. Initial t<sub>0.5</sub> values for the rates of decay following both substances were around 1.2 hours. It has been reported in *C. reinhardtii* that the adaptation process from low to high CO<sub>2</sub> conditions takes between 12 and 24 hours (see Badger, 1987), however Kaplan (1985) found in synchronously grown *C. reinhardtii* 2137 cells with a 12 h light and dark photoperiod that Ci transport was strongly reduced at the beginning of the dark period, which is consistent with the findings in Figure 3.10. The fact that the

Figure 3.10 : Effect of glucose and sodium bicarbonate addition on the *Ci* uptake mechanism in *Synechocystis* PCC6803





decay in the  $\text{Ci}$  uptake rate took place over such a short time span (less than 1 doubling time) suggested that gene control alone was not responsible for the modification seen in activity, since the predicted half-life of decay would be equivalent to half the growth rate (approx 8 hours), and some form of post-translational modification may have occurred to switch the  $\text{Ci}$  uptake mechanism off. One of the most widely studied areas of post-translational modification is protein phosphorylation, which is important in the control of many cellular processes including photosynthesis (see section 5.1 and in particular 5.1.4.4). This process has been studied in *Synechocystis* PCC6803 in response to growth under low and high  $\text{CO}_2$  and photoheterotrophic conditions in chapter 5.

It has been mentioned briefly that over the course of this study (approx 20 months) there was considerable variation in the  $\text{Ci}$  uptake rates seen in the same organism grown under identical growth conditions. This was not due to discrepancies in the methodology used, because the variation between identical samples in the same experiment was on average around 5-7 %. In the most extensively studied organism, *Synechocystis* PCC6803, "standard"  $\text{Ci}$  uptake assay conditions (100  $\mu\text{M}$   $\text{NaHCO}_3$ , pH 7.4, 30 s) on low  $\text{CO}_2$ -grown cells revealed that total  $\text{Ci}$  uptake rates varied from around 6.0 (see Figs. 3.6 and 3.8) to over 20 (see Figs. 3.9 and 3.10)  $\text{nmol}^{-1}\text{mg dry wt}^{-1} \text{ min}^{-1}$ . In all experiments the illumination and shaking rate were the same, and in each case the cells were harvested during mid-logarithmic phase.

Similar findings were also made with the rest of the organisms studied that possess a  $\text{Ci}$  uptake mechanism. This may in part be due to the variations in growth rates which inevitably occur in organisms grown in batch culture. However these variations were never on the same scale as the variations seen in the  $\text{Ci}$  uptake rate. This variation is thought to be due to variations which occur in the total DIC concentration in these batch cultures. A number of workers have reported that it is not  $\text{CO}_2$  concentration *per se*, but total DIC concentration that is responsible for induction of the high  $\text{CO}_2$  phenotype. This was initially reported by Miller et al. (1984a), working with chemostat cultures of *S. leopoliensis*. It was shown that cultures with an external  $\text{CO}_2$  concentration 6 times lower than that seen normally in solutions in equilibrium with air, still had a high  $\text{CO}_2$  phenotype because the total DIC in the chemostat was high. Mayo et al. (1986) and Badger and Gallacher (1987) found that batch cultures of *Synechococcus* PCC6301 grown over a range of different DIC concentrations produced cells with a continuum of  $\text{Ci}$  uptake affinities. Badger and Gallacher found that cells with the highest affinity for  $\text{Ci}$  could only be obtained when the external DIC concentration was less than 50  $\mu\text{M}$  and that cells with the lowest affinity for  $\text{Ci}$  at an external DIC concentration of 2 mM. Between these two extremes cells with intermediate affinities for  $\text{Ci}$  were found. Similar results were obtained by Mayo et al. (1986). It is believed that the variation in the results obtained in the series of experiments conducted in this study are the result of the external DIC

concentration in the medium producing low CO<sub>2</sub>-grown cells with varying affinities for Ci. It was not possible to standardise the external DIC concentration in batch cultures even though the rate of shaking was kept the same. Badger and Gallacher (1987) state that production of cells in batch culture with a high affinity for Ci by growing on air "has been fortuitous" and would only be achieved by low bubbling rates and high Ci concentrations. It is believed that the variations seen in high CO<sub>2</sub>-grown cultures throughout these experiments was similarly the result of producing cells with a continuum of affinities for Ci. In order to avoid these problems, the Ci uptake process was studied in chemostat cultures, where defined DIC concentrations could be achieved (see chapter 4).

### 3.4 Conclusions

This chapter has shown that the doubts cast over the use of vacuum filtration for studying Ci uptake (see Miller 1990) have proven unfounded, as consistent results were produced, and similar rates of Ci uptake were seen to those observed by other authors. Silicone oil centrifugation using cells labelled with tritiated leucine enabled this technique to be more readily quantified, however it did not produce results which were any more consistent than vacuum filtration and so was abandoned in favour of this much simpler and quicker method. The facilities needed to carry out MIMS were not available.

There was no correlation found between RuBisCO activities and the ability of the cells to transport Ci. In most species



studied RuBisCO activities were similar in high and low CO<sub>2</sub>-grown cells and where differences occurred, activities were higher in high CO<sub>2</sub>-grown cells. On the contrary, Ci uptake and concentration rates were, in those species of cyanobacteria which possessed an active Ci uptake mechanism, always higher in low CO<sub>2</sub>-grown cells. This was true throughout a range of external pH's from 5.95 to 9.5, however under acidic conditions uptake rates were similar in cells isolated from both growth conditions.

The most striking features of the work in this study were the absence of a Ci uptake mechanism, under the conditions employed, in the oceanic *Synechococcus* sp., and the decay in the Ci uptake rate of *Synechocystis* PCC6803 caused by glucose and the non-metabolizable analogue DOG. The absence of a Ci uptake and concentrating mechanism in the oceanic *Synechococcus* has been discussed in relation to the size, growth rates, and most importantly environment these organisms are naturally found in. With a constant 2 mM DIC concentration in the external environment and relatively slow growth rates, the selection pressure to develop and maintain such a system is questioned. The decay in the Ci uptake rate of low CO<sub>2</sub>-grown *Synechocystis* PCC6803 upon addition of glucose (and bicarbonate) was very rapid with  $t_{0.5}$  values for the decay rate of the Ci uptake mechanism of 1.2 hr, suggesting that gene control alone is not responsible for the decay. Addition of the glucose analogue OMG at the same concentration as DOG to low CO<sub>2</sub>-grown cells did not lead to a decay in the Ci uptake mechanism. It is suggested that

build-up of a pool of glucose-6-phosphate is a signal for the switch-off of the  $C_i$  uptake mechanism, and that due to the subtle structural differences between DOG and OMG, DOG can enter the active site of hexokinase and become phosphorylated whilst OMG, because of its methyl substitution cannot.

## Chapter 4

Growth, inorganic carbon transport and membrane  
isolation in DIC and light-limited chemostat cultures  
of *Synechococcus* PCC7942



#### 4.1 Introduction

##### 4.1.1 The 42 kD cytoplasmic membrane polypeptide

In the early 1980's a number of workers separated the different membrane components of unicellular cyanobacteria (see Omata and Murata, 1983; Omata and Murata, 1984; Resch and Gibson, 1983). In 1985 it was found that there was a marked increase in a 42 kD polypeptide in the cytoplasmic membrane of *Anacystis nidulans* R2 during adaptation of high CO<sub>2</sub> grown cells to low CO<sub>2</sub> conditions (Omata and Ogawa, 1985), and further work by Omata and Ogawa (1986) led these authors to suggest that this 42 kD polypeptide was involved in active Ci transport by *Anacystis nidulans* R2 and its synthesis under low CO<sub>2</sub> conditions lead to high Ci transporting activity. Further immunochemical studies showed that an antibody raised against the 42 kD cytoplasmic membrane polypeptide from *Anacystis nidulans* R2 reacted with a 45 kD polypeptide from the cytoplasmic membrane of low CO<sub>2</sub> adapted *Synechocystis* PCC6803 (Omata and Ogawa, 1987). In the same study the authors found that the antibody raised against the 42 kD protein did not react with any proteins in high CO<sub>2</sub> adapted *A. nidulans* R2 and *Synechocystis* PCC6803. They suggested the following possibilities regarding the role of the 42 kD (or 45 kD in *Synechocystis*) polypeptide.

- i) All the machineries necessary for Ci transport are present in high CO<sub>2</sub>-grown cells. The 42 kD protein is essential to achieve their full activity.
- ii) The Ci transporting system in high CO<sub>2</sub> grown cells is different from that induced in low CO<sub>2</sub> cells, the 42 kD

polypeptide functioning in the low CO<sub>2</sub> induced system.

Although it has since been proven that the role of the 42 kD protein is not directly concerned in CO<sub>2</sub> uptake (Schwarz et al., 1988, Omata et al., 1990), early work in this study was assigned to determining a role for this protein in *Synechococcus* PCC7942.

#### 4.1.2 Chemostat cultures

Most of the work in the previous decade on the Ci concentrating mechanism in cyanobacteria has been carried out in batch culture systems. In these batch culture systems the actual form and concentration of the DIC in the medium are dependent on the bubbling rate, pH and the algal photosynthetic rate. Karagouni and Slater (1979) used chemostats to determine the effect of carbon limitation on enzyme activities in *Anacystis nidulans*, but gave no information on the DIC concentrations at the various growth rates. More detailed analysis of the Ci concentrating mechanism under continuous-flow culture was performed by Miller et al. (1984a) and Turpin et al. (1985b) with the same organism as used by Karagouni and Slater (1979). A chemostat is a continuous-flow culture in which the growth of the organism is limited by a single resource. The system enables the growth rate to be varied whilst maintaining a constant environment, or conversely the environment can be varied whilst the growth rate is kept constant. Growth conditions can be established, allowing the study of the physiology of the organism under different substrate limiting conditions, which may be extremely important for the growth of the organism in the natural environment.

Continuous-flow culture systems have a special advantage over batch culture systems in enzyme regulation studies. Once a batch culture has been established, there is no further input or output of materials and the process is discontinuous because the environment changes continuously as a result of the organism's growth. In contrast, steady state conditions can be obtained in continuous-flow cultures, whilst environmental conditions and biomass concentration remaining constant, and so critical quantitative changes in enzyme levels and activities can be made in organisms growing under steady state conditions, and many workers have endorsed the advantages of chemostat culture systems in the study of enzyme regulation (see Karagouni and Slater, 1979).

#### 4.1.3 Theory of chemostat operation

The following section provides a brief review into the operation of, and pertinent theory of chemostat cultures, however a much more detailed discussion of the theoretical treatments of chemostat cultures can be obtained by referring to the texts of Herbert et al. (1956) and Pirt (1975).

Several criteria must be fulfilled to enable the development of correct chemostat operation in cyanobacteria. These are:

- (1) The chemostat must be well mixed to ensure a homogeneous environment.
- (2) Cell loss through culture outflow must be non-selective
- (3) Cell viability is independent of growth rate
- (4) A single nutrient is growth limiting



(5) Light intensity, except in light limited chemostats, is growth saturating.

In a chemostat, fresh medium is continuously pumped into a well mixed culture vessel of fixed volume ( $V$ ), at a constant flow rate ( $F$ ), and an equal volume of culture is removed. The organism is grown at specific growth rates ( $\mu$ ) which are less than the maximum specific growth rate ( $\mu_{max}$ ). The concentration of the growth-limiting substance in the chemostat depends both on the rate at which fresh substrate is supplied and on the dilution factor as fresh substrate is dispersed throughout the culture vessel. Exponential biomass increase is opposed by the process of continual culture dilution. The dilution rate ( $D$ ) of the chemostat is directly proportional to the flow rate of sterile medium into the reactor and inversely proportional to the volume of the reactor, such that

$$D = F/V \quad (4.1)$$

The dilution rate has units of reciprocal time, usually  $h^{-1}$ , and is a measure of the number (or fraction) of the culture volume changes achieved in unit time. The mean residence time of cells in the reactor can be calculated as  $1/D$ .

As the incoming medium, with an initial concentration of growth-limiting substrate ( $S_0$ ) is mixed into the culture, some of it is used to produce fresh biomass, which has a concentration of  $x$ , the concentration of the growth-limiting substrate being reduced to  $s$ .

Cell densities are determined by two competing processes, growth

and dilution. Within the culture vessel there is a balance such that the change of biomass concentration is equal to the rate of growth minus the rate of biomass washout.

$$\text{Thus } dx/dt = ux - Dx$$

$$dx/dt = x (u-D) \quad (4.2)$$

$$\text{biomass change} = \text{growth} - \text{loss}$$

with reference to equation (4.2) there are three different possible situations to be considered:

(a) If  $u > D$ , then  $dx/dt$  is positive and biomass concentration in the growth vessel increases since the rate of biomass production exceeds the rate of biomass washout.

(b) If  $u < D$ , then  $dx/dt$  is negative and biomass concentration will decrease since the rate of culture washout exceeds biomass synthesis.

(c) If  $u = D$ , then  $dx/dt = 0$  and biomass concentration remains constant, a steady state culture having been obtained. As growth rate under steady state conditions is dependent upon addition of the growth limiting substrate (and hence upon dilution rate) the steady state growth rate of cells in chemostat culture at all dilution rates must be less than maximal.

Monod (1942) showed that there was a simple relationship between the specific growth rate ( $u$ ) and the concentration of an essential growth substrate ( $s$ ),  $u$  being proportional to the substrate concentration according to the equation:

$$u = u_{\max} \frac{s}{K_s + s} \quad (4.3)$$

where  $K_s = s$  at  $u = 1/2 u_{max}$  and  $s^*$  = the steady state substrate concentration. In the case of this study, where Ci-limited chemostats are in operation,  $s = [DIC]^*$  (the steady-state DIC concentration).

Turpin et al. (1985a) present a summary of  $K_s$  values for a range of algal species under DIC-limited growth, and in most cases they are in the low micromolar range.

From equation (4.2) it can be seen that there is an upper limit to the dilution rate above which steady state cultures cannot be established. This is because the organism's specific growth rate has a maximum value,  $u_{max}$ , which is genetically determined, and cannot be exceeded. Thus, if  $D > u_{max}$  a steady state culture cannot be maintained and the culture is said to washout. The highest dilution rate at which steady state conditions can be maintained is known as the critical dilution rate,  $D_{crit}$ . This occurs in DIC limited chemostats when the DIC concentration in the reactor,  $[DIC]$ , approaches the highest possible value, approximately equal to the inflowing inorganic carbon concentration.

#### 4.1.4 DIC-limited chemostat cultures

Carbon generally accounts for between 40-50% of dry algal biomass. The macronutrients C, N and P are required in a relative molar concentration ratio of 106:16:1 (Goldman et al., 1979), the Redfield ratio. Algae growing in media with a C:N and C:P ratio greater than 6.6 and 16 respectively will be limited by DIC, however in practice non-limiting nutrients are generally



added at concentrations greatly exceeding those dictated by the Redfield ratio.

It has been shown by a number of workers that the relationship between cellular biomass and cell carbon is independent of the degree of DIC limitation (Goldman and Graham, 1981; Miller et al., 1984a), which is not the case for nitrogen (Goldman and McCarthy, 1978) and phosphorous (Goldman and Rhee, 1981), which should in principle make the study of DIC-limited growth easier. DIC limitation in the chemostat can be shown by observing a proportional increase in steady-state biomass corresponding to increased concentrations of the limiting nutrient in the inflow, as in this study, or observing a transient increase in biomass in response to an inoculation of DIC into the chemostat (Miller et al., 1984a).

Karagouni and Slater (1979) showed that the activities of a number of enzymes of the reductive and oxidative pentose phosphate and glycolytic pathways varied significantly as a function of the growth environment and organism growth rate. In  $\text{CO}_2$ -limited cultures they found that the specific activity of RuBisCO, both in terms of activity per unit protein or per cell number, increased 15-fold with decreasing dilution rate. The specific activity was constant at dilution rates above  $0.10 \text{ h}^{-1}$  and only increased when the dilution rate was below this figure. Under light-limited conditions, with  $\text{CO}_2$  in excess, the specific activity of RuBisCO remained constant at all dilution rates, leading the authors to conclude that the increased specific activity of RuBisCO was in response to changes in external  $\text{CO}_2$ .

concentrations, which would increase the capacity to the enzyme to scavenge for and assimilate low, growth-limiting concentrations of the sole carbon source.

Miller et al. (1984a) have shown in Ci-limited chemostats of *Synechococcus leopoliensis* that at growth rates below  $1.7\text{d}^{-1}$ , essentially all the supplied Ci was converted to organic carbon and the cells were carbon limited. These carbon-limited cells used  $\text{HCO}_3^-$  rather than  $\text{CO}_2$  for growth and  $\text{O}_2/\text{CO}_2$  ratios were as high as 192,000:1. Their studies also revealed that in this organism, induction of the "high  $\text{CO}_2$  syndrome" (where the Ci concentrating mechanism is absent or has a greatly reduced capacity to transport Ci) did not actually require high  $\text{CO}_2$ , but only high [Ci], and was seen even when the steady-state  $\text{CO}_2$  concentration in the chemostat was lower than that seen in solutions equilibrated with air.

Although it has been shown in batch culture that growth on intermediate levels of DIC results in cells with intermediate affinities for DIC (Mayo et al., 1986; Badger and Gallacher, 1987) in chemostat cultures a number of workers have shown that there is a sharp transition in the affinity of cells for DIC, with only fully induced or fully repressed cells being present (Miller et al., 1984a; Turpin et al., 1985b; Mayo et al., 1986). Turpin et al. (1985b) found that chemostat cultures of *S. leopoliensis* fell into two distinct categories with respect to the carbon-dependence of short-term photosynthesis and a single Monod relationship could not be used to describe the  $\mu$  vs [DIC]<sup>\*</sup> relationship for DIC-limited cultures. In DIC-limited cultures

growing at growth rates below  $1.7 \text{ d}^{-1}$ , with steady-state  $[\text{DIC}]^*$  below  $100 \text{ }\mu\text{M}$  they found that the Monod expression was a physiologically valid representation of  $[\text{DIC}]$ -limited growth, however as  $\mu$  approached  $\mu_{\text{max}}$  in cultures with steady-state  $[\text{DIC}]^*$  above  $2 \text{ mM}$ , the cultures displayed multiphasic kinetics. However, the validity of the methodology used must be questioned since all of these authors have used the methodology of Miller et al. (1984a) who quite clearly state that light was not limiting at any growth rate and yet do not state what substrate was limiting in "carbon sufficient" cultures. In addition, these experiments were performed in a chemostat in which culture volume was only  $100 - 110 \text{ ml}$ , and in some cases up to  $50 \text{ ml}$  of cells were required for a given experiment, which could lead to problems maintaining steady-state during sampling.

The aims of the work performed in continuous-flow culture for this thesis were to study  $\text{Ci}$  transport and  $\text{CO}_2$  assimilation in *Synechococcus* PCC7942 under conditions of known  $\text{CO}_2$  supply, and to assess and correlate the modulation of the  $42 \text{ kD}$  polypeptide under these conditions with the  $\text{Ci}$  concentrating mechanism.



### 4.3 Results and Discussion

#### 4.3.1 Isolation and characterization of membranes from

##### *Synechococcus* PCC7942

Floatation centrifugation on sucrose gradients separated several membrane fractions from cell homogenates of *Synechococcus* PCC7942 (Fig. 4.1).

Cell homogenates were prepared from high and low CO<sub>2</sub>-grown cells, and the gradients obtained following floatation centrifugation were fractionated (2ml fractions) by inserting a 21 gauge needle into the base of the gradient. These fractions were then subjected to spectroscopy in a LKB Ultraspec II over a range of different wavelengths to determine the composition of the various fractions.

The result of this analysis on both the high and low CO<sub>2</sub> membrane fractions is shown in Figure 4.2A and 4.2B. Both high and low CO<sub>2</sub> fractions showed a very similar trend with respect to absorbance v fraction number. In most of the sucrose gradients obtained by the floatation centrifugation method, there was no clear demarcation between the various membrane fractions, and this is clearly shown in Fig. 4.1. There is a diffuse orange band stretching from the top of membrane fraction T, to the bottom of membrane fraction C. The spectrometric analysis followed a similar pattern to that seen in the sucrose gradient. Unbroken cells and large pieces of cellular debris pelleted at the bottom of the gradient, whilst in the 50% sucrose layer there is a great deal of protein and pigment (all readings were performed on samples diluted 1:10). The 50%

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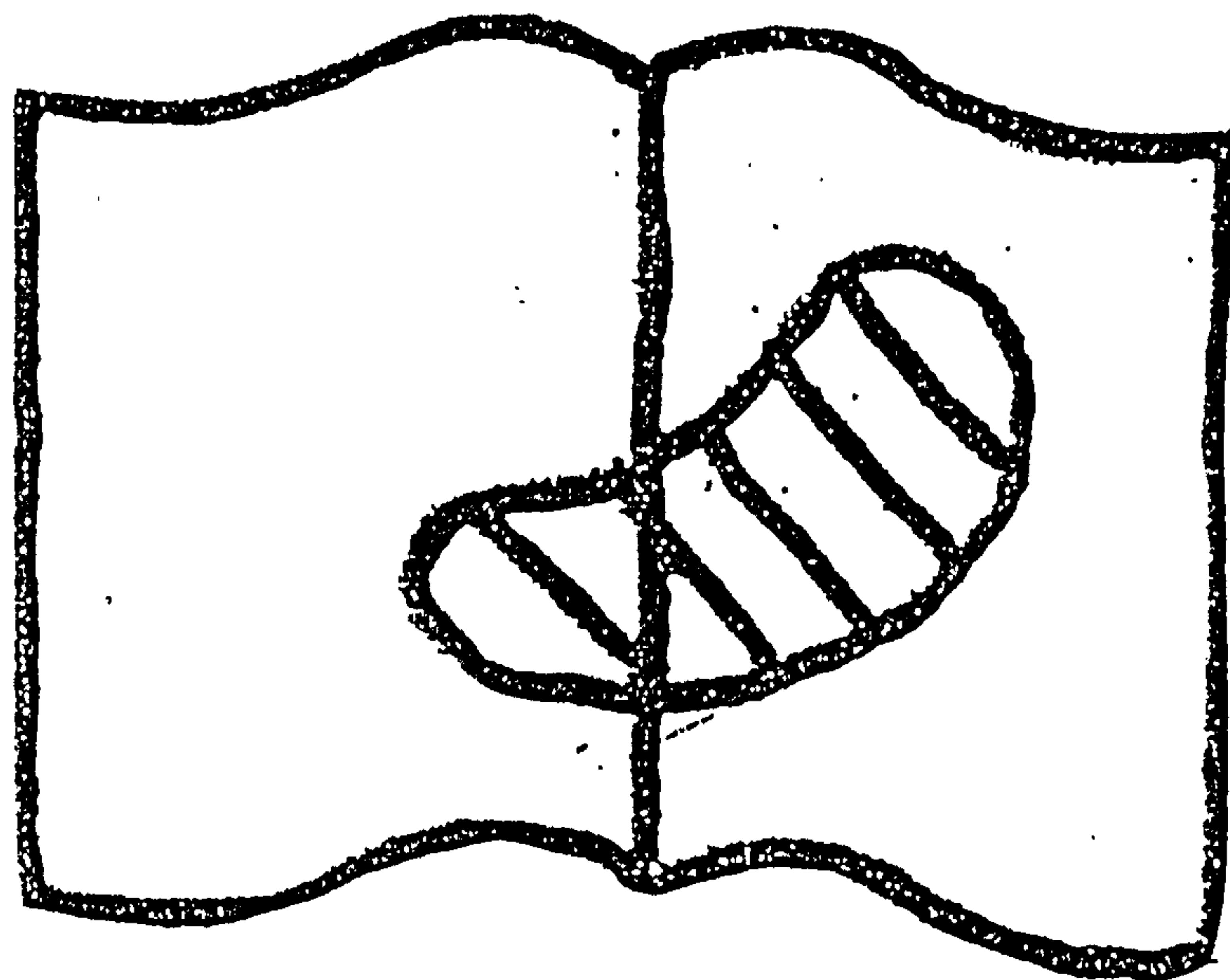




Figure 4.1 : *Synechococcus* PCC7942 membrane fractions

A) Sucrose gradient obtained following floatation centrifugation of a cell homogenate of *Synechococcus* PCC7942. C = cytoplasmic membrane fraction, M = mixed membrane fraction (cytoplasmic and thylakoid), T = thylakoid membrane fraction.  
 B) Absorption spectra of isolated membranes.

**A**

C

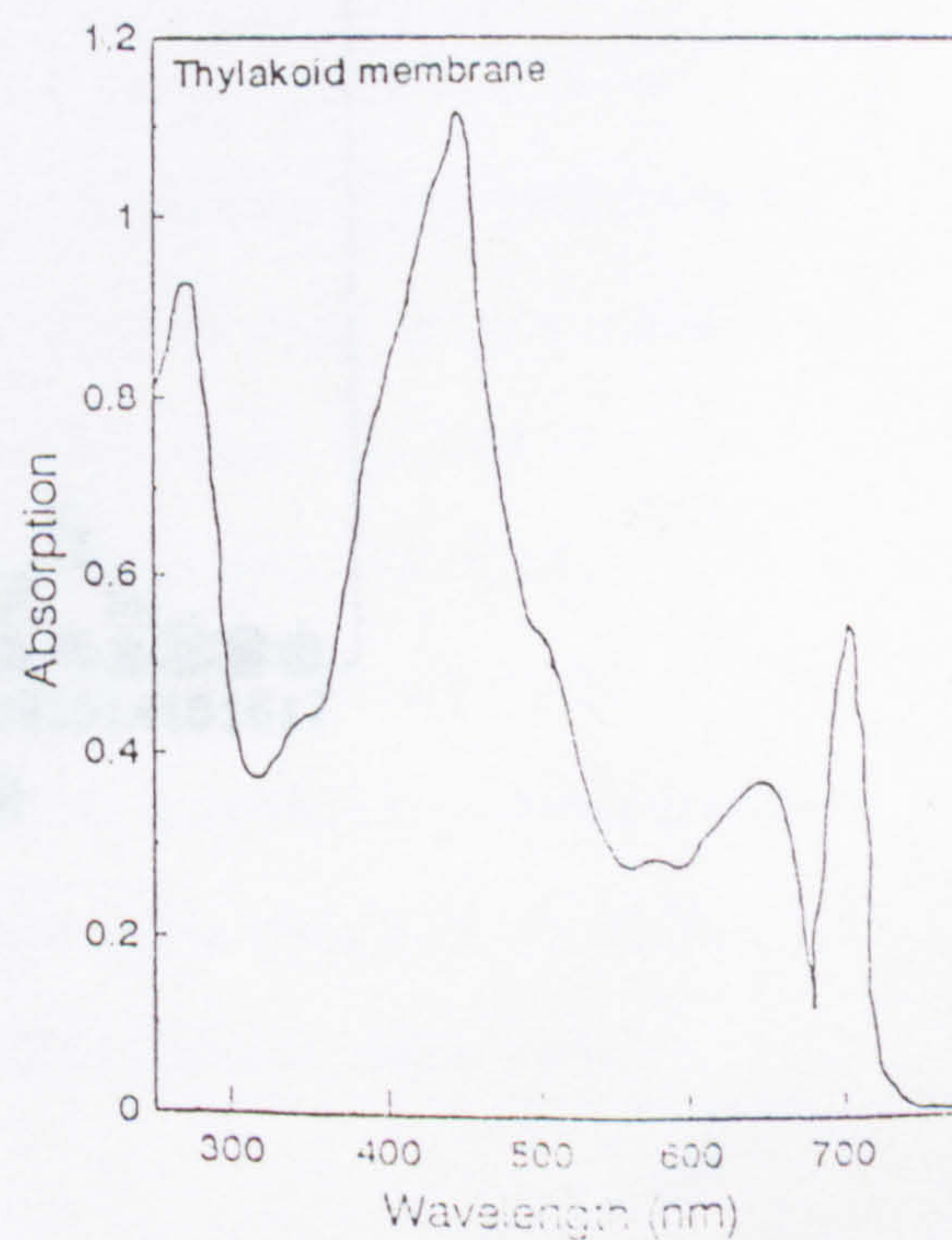
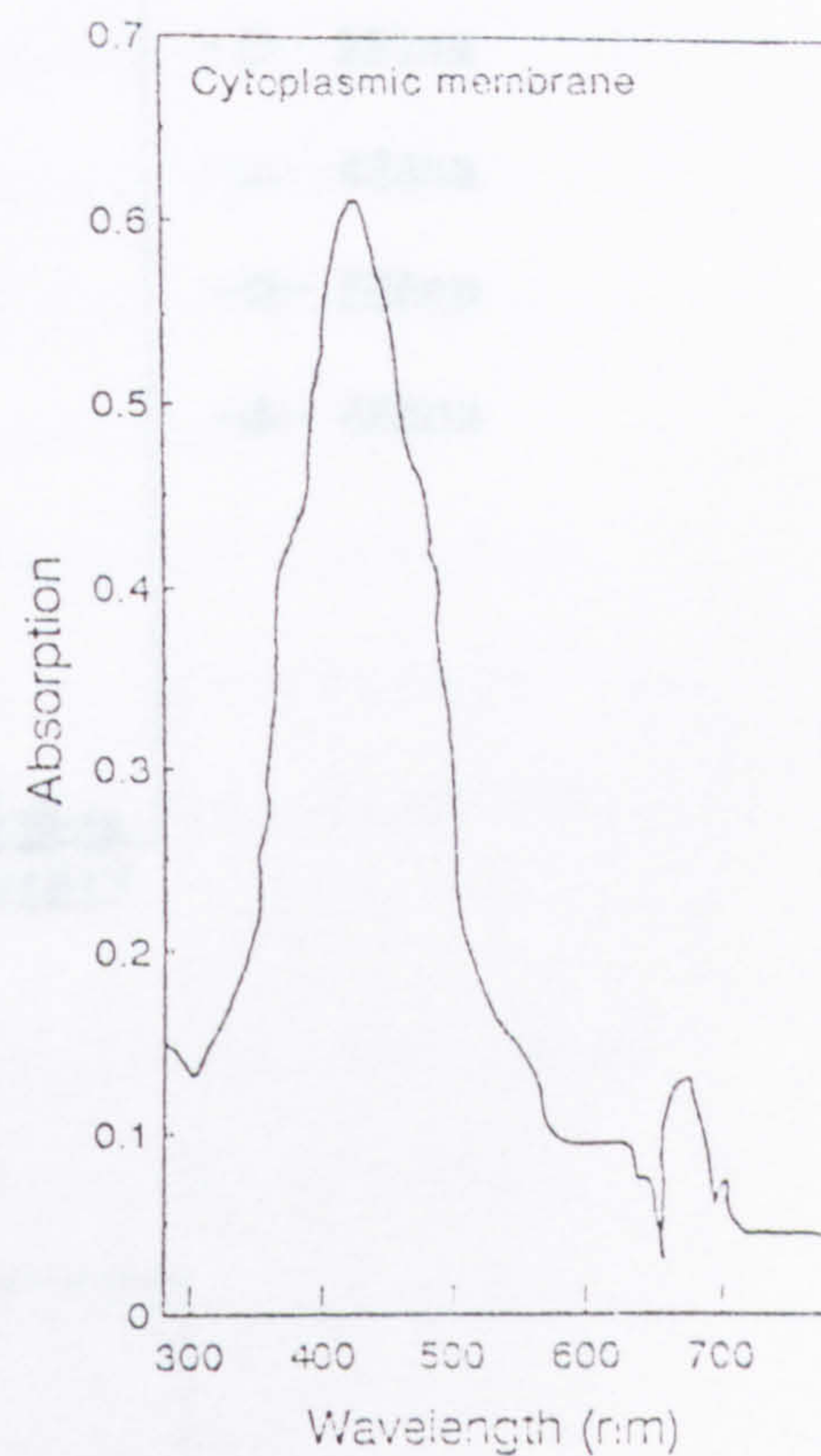
M  
T**B**



Fig. 4.2 : Absorption spectroscopy of fractionated sucrose gradients

Fig. 4.2A : Low  $\text{CO}_2$  gradient

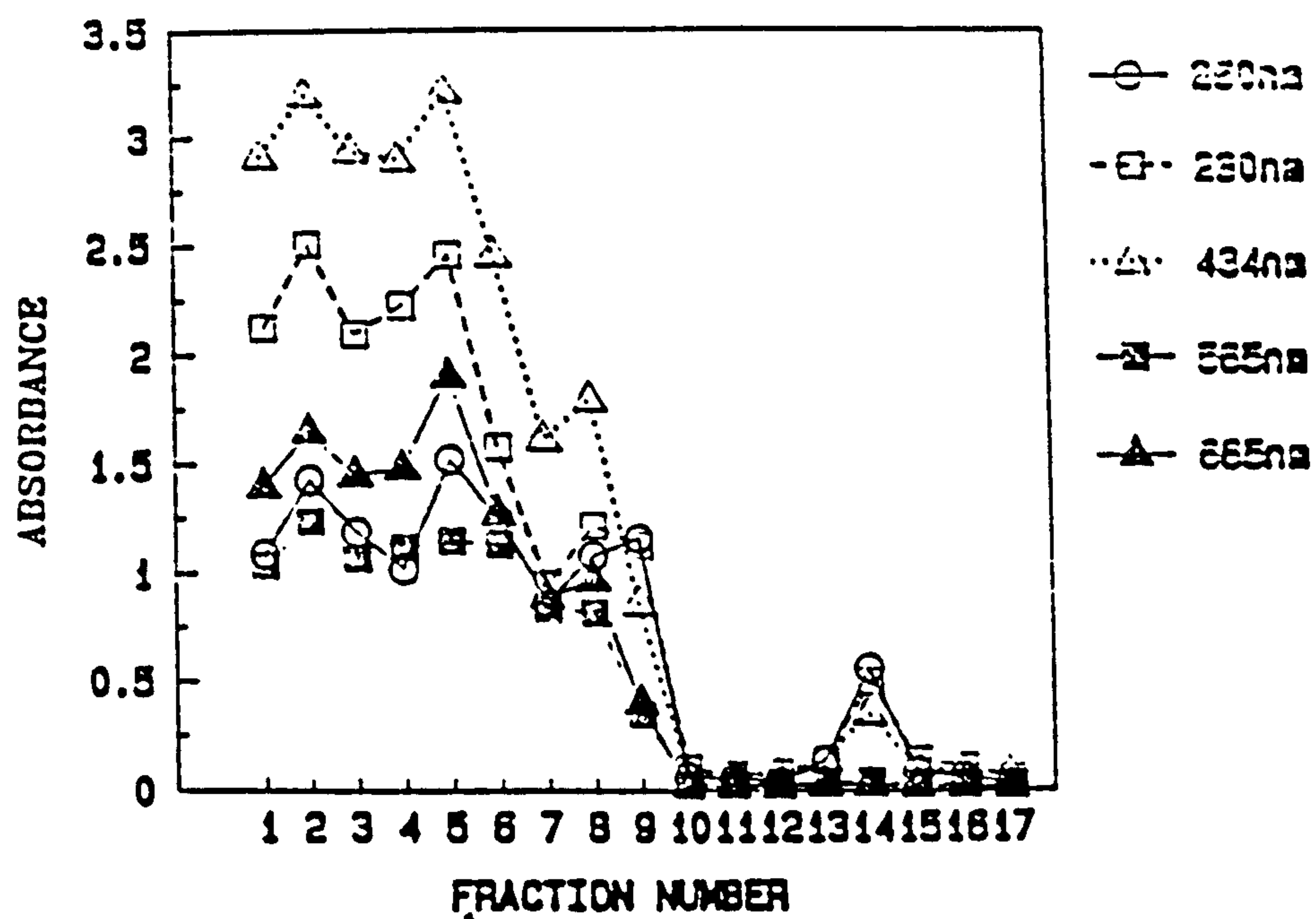
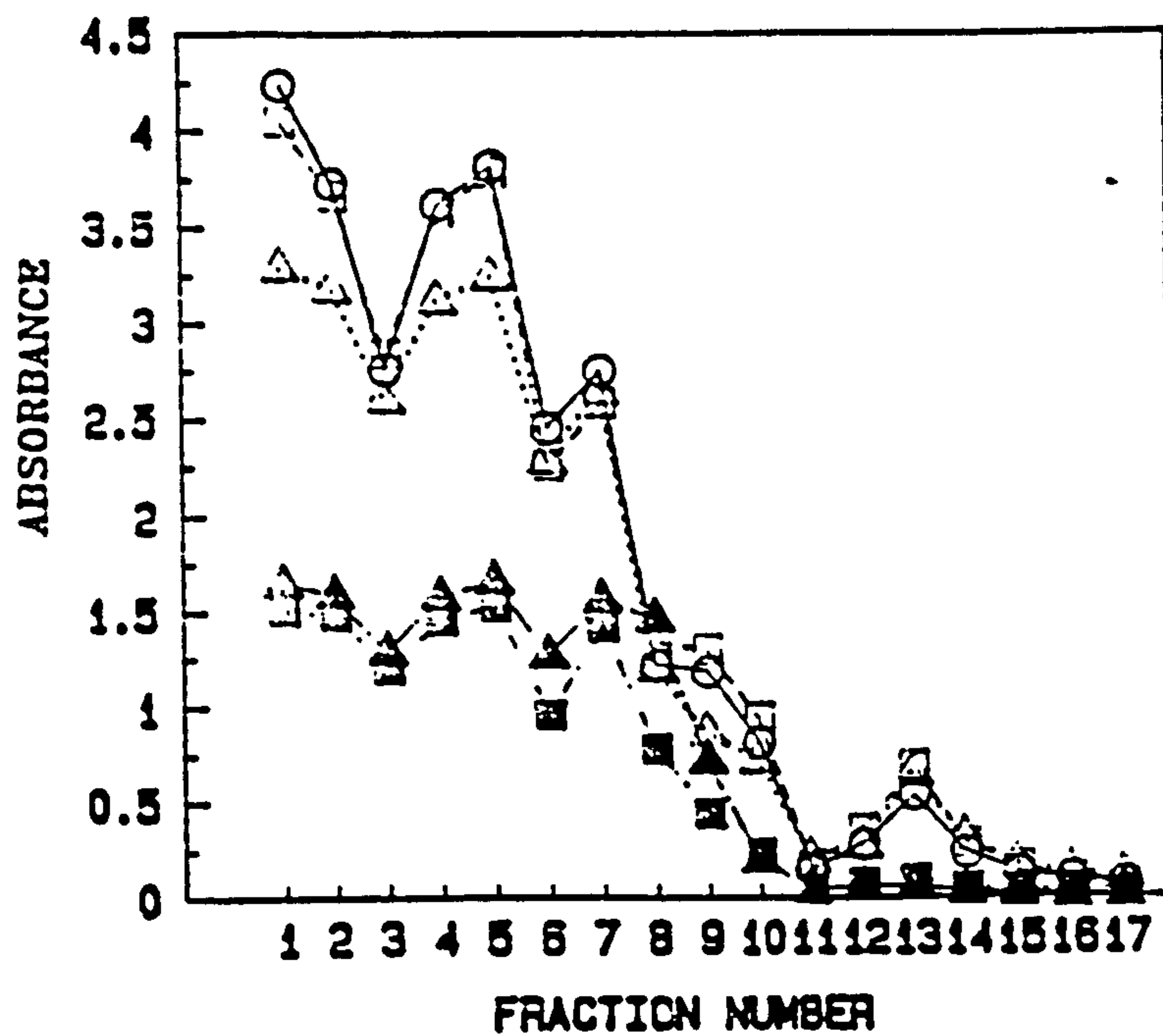


Fig. 4.2B : High  $\text{CO}_2$  gradient



sucrose layer was a deep purple colour, and obviously contained most of the contents of the cell (excluding cytoplasmic and thylakoid membranes).

The fractions of most interest are fractions 8 and 9, where the dark green fraction banded, and fractions 14 and 15, which represent the main orange band seen on the gradient. In fractions 8 and 9, which is probably the thylakoid membrane fraction of Omata and Murata (1983), there are large amounts of carotenoid and the light harvesting pigments, phycocyanin, allophycocyanin and chlorophyll a, and this fraction contains per unit amount of protein, the largest amount of chlorophyll a, which is as we would expect, as chlorophyll a is localised in the thylakoid membrane.

After fraction 9 the protein content in each fraction decreased significantly until fractions 13 and 14. The low CO<sub>2</sub> gradient showed a much clearer demarcation between the main membrane fractions (not shown) than the high CO<sub>2</sub> gradient, and consequently these fractions contained much less of the various components being measured. In fractions 13 and 14, both the protein and carotenoid content increased, however, phycocyanin, phycoerythrin and chlorophyll content in these two fractions remained very low, suggesting that this fraction is the cytoplasmic membrane fraction of Omata and Murata (1983). Absorption spectroscopy at 28°C of the membrane fractions obtained from these gradient (see Fig. 4.1) revealed absorption spectra similar to those obtained by Omata and Murata (1983), confirming that fraction (C) was the cytoplasmic membrane

fraction, fraction (T) the thylakoid membrane fraction and fraction (M) a mixture of thylakoid and cytoplasmic membrane fractions. The major peak at 445nm with shoulders at 390 nm and 480nm in *Synechococcus* PCC7942 represented carotenoid absorption. Of the remaining peaks, that at 680nm was due to chlorophyll a, whilst that at 628nm was probably due to phycocyanin absorption. Using an Abbe refractometer (Bellingham and Stanley Ltd), it was possible to determine the sucrose concentration and buoyant density ( $\text{g/ml}^{-1}$ ) each membrane fraction banded at. *Synechococcus* PCC7942 fraction (C) banded at a sucrose concentration of 27% (1.1108) and fraction (T) at a sucrose concentration of 42% (1.1848) [*Synechocystis* PCC6803 fraction (C) banded at a sucrose concentration of 24% (1.097) and fraction (T) at a sucrose concentration of 41% (1.1797)]. Table 4.1 shows the result of various protein determination methods on cytoplasmic membrane fractions isolated from sucrose gradients following flotation centrifugation. With the exception of the 280/260 nm spectrophotometric analysis, which was performed on samples straight from the sucrose gradient, the other protein determinations were performed on pelleted membrane fractions, resuspended in 10 mM Tris-NaOH. By analysis of the amount of protein loaded onto a polyacrylamide gel (data not shown) it was found that the most accurate method for determining the amount of protein present in the various membrane fractions was that of Lowry et al. (1951), provided the samples were boiled for 5 minutes to hydrolyse the proteins. The BioRad microassay method (BioRad laboratories) proved to be



Table 4.1 : Quantitative analysis of cytoplasmic membrane fractions from Synechococcus PCC7942

Cytoplasmic membrane	Protein determination method			
	-----			
Sample number	Lowry	Lowry (Boiled)	BioRad micro	280/260 spectro
1	1.3	0.64	0.70	0.714
2	1.26	0.36	0.40	0.748
3	1.32	0.44	0.40	0.745
4	1.10	0.20	0.18	0.242
5	1.84	0.16	0.13	0.195
6	1.26	0.22	0.18	0.502
7	1.70	0.23	0.20	0.376
8	1.38	0.14	0.15	0.217
9	1.46	0.69	0.60	0.764
10	1.25	0.30	0.28	0.413

almost as accurate, whilst the Lowry method (no prior boiling of samples and the 280/260 nm spectrophotometric method were the least accurate (overestimation). The BioRad microassay was the most widely used method for determining protein loading for PAGE because of the simplicity and speed of the method and the fact that only a small amount (20ul) of sample was required.

SDS-PAGE (7.5-20% exponential) of the sucrose gradient fractions from high and low CO<sub>2</sub>-grown *Synechococcus* PCC7942 revealed that under both growth conditions a majority of the polypeptides are found in every membrane fraction (see Figs. 4.3A and 4.3B respectively). Omata and Murata (1983) reported that there were no polypeptides common to fraction C and fraction T. Although it is likely that the 50% sucrose layer will contain contaminating protein from all of the cell's membrane fractions, the fact that every fraction from both high and low CO<sub>2</sub> gradients contained most of the polypeptides suggested that rather than being "common" cell polypeptides, by fractionating the gradient from the bottom, contamination of the topmost gradient fractions with those from the lower layers of the gradient has occurred. However, when Fig. 4.3A and Fig. 4.3B are compared (each gel had the same protein loading per track) it can be seen there are differences in the polypeptide composition from the two growth regimes. In particular, if the fractions corresponding to the cytoplasmic membrane fraction are compared (fractions 14 and 15 in Fig. 4.3A and 13 and 14 in Fig. 4.3B) it can be seen that there is a polypeptide Mr 30,000, seen under both growth



Figure 4.3 : SDS-PAGE of sucrose gradient fractions from high and low CO<sub>2</sub>-grown *Synechococcus* PCC7942

4.3A Low CO<sub>2</sub> gradient

Fraction No. 1 4 7 9 10 11 12 13 14 15 16 17 18

$M_r \times 10^3$

92

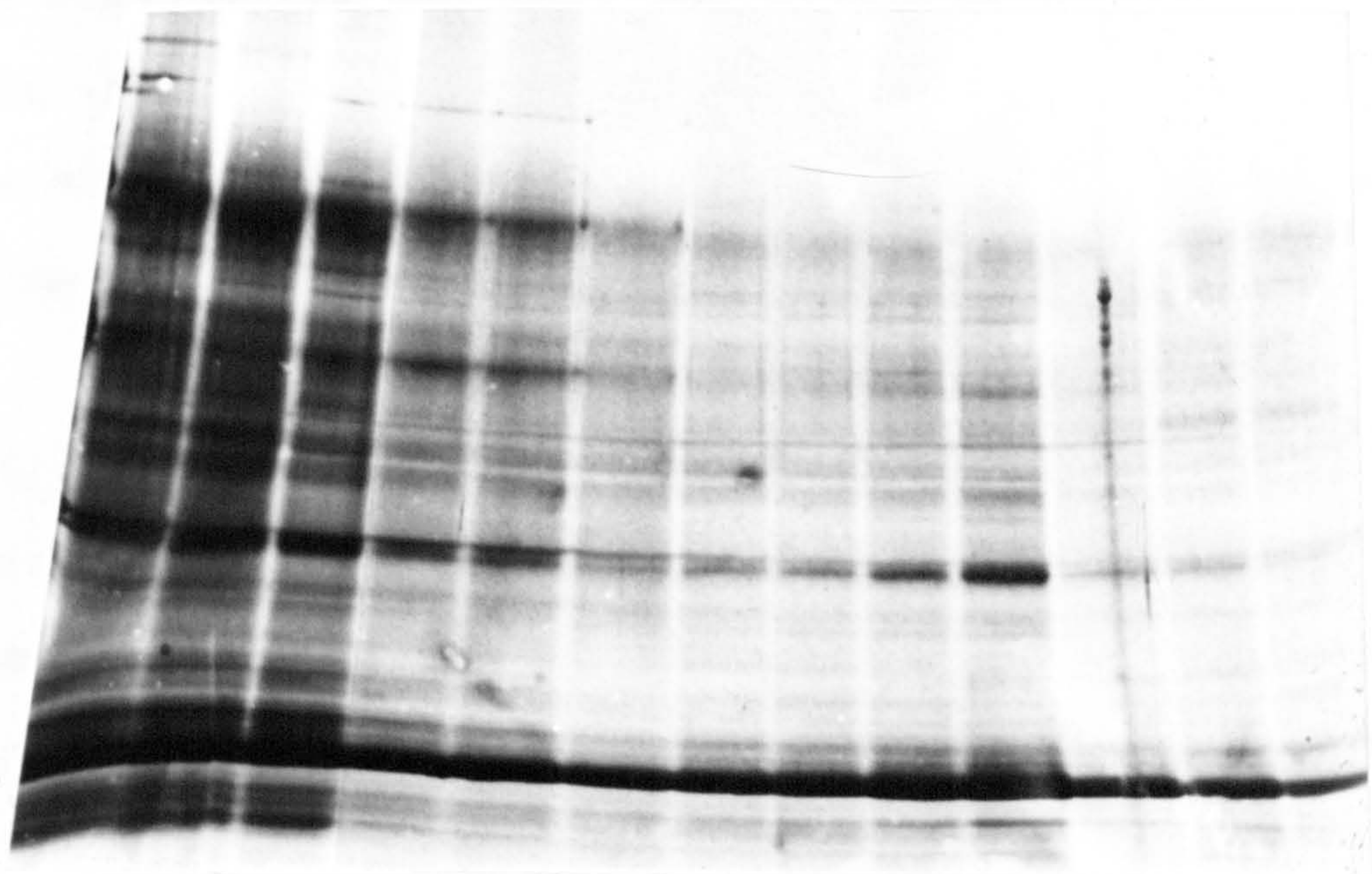
67

43

30

20

14.4



4.3B High CO<sub>2</sub> gradient

Fraction No. 1 3 5 6 7 8 9 10 11 12 13 14 15

$M_r \times 10^3$

92

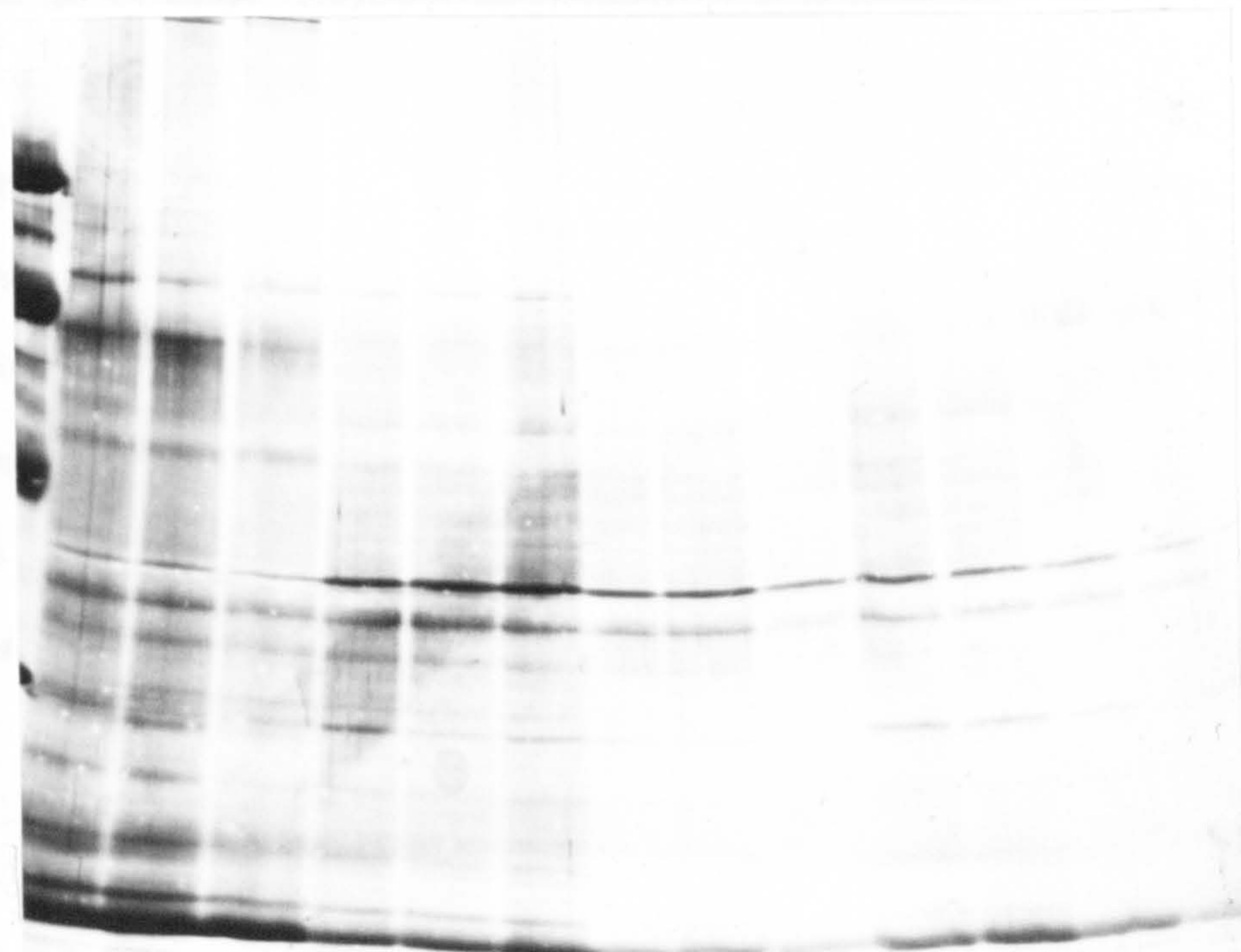
67

43

30

20

14.4





conditions, that is far more abundant in cells grown under low CO<sub>2</sub> conditions. There are also two polypeptides of Mr 35,000 and 37,000 which are more abundant in Fig. 4.3B and another from Fig. 4.3A of Mr 43,000, which although not particularly abundant in fractions 14 and 15, is not seen in Fig. 4.3B. The identity of most of these polypeptides can only be guessed at, however the Mr 43,000 polypeptide may well be, allowing for irregularities in the migration pattern of proteins during SDS-PAGE, the 42 kD cytoplasmic membrane polypeptide reported by Omata and Ogawa (1985 & 1986).

#### 4.3.2 Effects of various treatments on cytoplasmic membrane yield in *Synechococcus* PCC7942 and *Synechocystis* PCC6803

As can be seen from Table 4.2, the yield of cytoplasmic membrane from the sucrose gradient is very small, varying between 0.018 and 0.047% of the total protein loaded onto the sucrose gradient. As it was intended to purify large enough quantities of this membrane fraction to allow isolation of the 42 kDa polypeptide for antibody production, a variety of pretreatments of the cells were tried, prior to French pressing, to try and improve yields of the cytoplasmic membrane fraction. Table 4.3 shows the effect of various pretreatments on cytoplasmic membrane yields in *Synechocystis* PCC6803. As can be seen incubation with lysozyme prior to French pressing improved the yield of the membrane fraction, which is obviously due to the effect of this enzyme on the cell wall, resulting in a greater breakage of the cells upon French pressing. However, there were

Table 4.2 : Cytoplasmic membrane yields from Synechococcus PCC7942 and Synechocystis PCC6803

Organism	Dry weight (mg)	Total protein (TP) (mg)	Membrane protein (MP) (ug)	$\frac{MP}{TP}$ (%)
7942 L	240	120	47	0.039
7942 H	352	176	83	0.047
6803 L	448	224	48	0.020
6803 H	560	280	51	0.018
6803 HET	368	184	49	0.027

Table 4.3 : The effect of various pretreatments on cytoplasmic membrane yields in Synechocystis PCC6803

Treatment	TP (mg)	Protein loaded onto gradient (mg)	Membrane protein recovered (ug)	Yield (%)
No Lysozyme	280	120	28.0	0.022
Lysozyme 1mg/ml 2hrs	280	240	50.0	0.021
Lysozyme 3mg/ml 2hrs	280	220	49.0	0.023
NaI <sub>2</sub> wash + Lysozyme 3mg/ml 2hrs	280	240	49.5	0.020

no improvements in yield following incubation with larger amounts of lysozyme, suggesting that even at 1 mg/ml it is present in excess. There was also no improvement in yield when the cells were given an  $\text{NaI}_2$  (2 mg/ml) wash prior to incubation with lysozyme.  $\text{NaI}_2$  is supposed to help break down the exopolysaccharide coat found around *Synechocystis* PCC6803 (A. Turner per. comm.) allowing the lysozyme to work more effectively on the cell wall, however from results obtained this did not prove to be the case. As expected the greatest yields were obtained from cells in mid - late logarithmic phase, once cells reached stationary phase they proved harder to break and yields of the various membrane proteins were correspondingly smaller (data not shown).

As there were no improvement in yield following these various pretreatments, the only effective way to produce larger amounts of cytoplasmic membrane was to "bulk up" production by using 5 and 10 l, instead of 1 or 2 l, flasks.

#### 4.3.3 Further characterisation of membrane fractions from *Synechococcus* PCC7942

To establish whether or not the polypeptide seen at Mr 43,000 was indeed the 42 kDa cytoplasmic membrane polypeptide, additional sucrose gradients using cell homogenates from low and high  $\text{CO}_2$ -grown *Synechococcus* PCC7942 were subjected to floatation centrifugation. After floatation centrifugation the various membrane fractions were separated, pelleted and approximately 20 ug of each sample run in parallel on a 10-30%



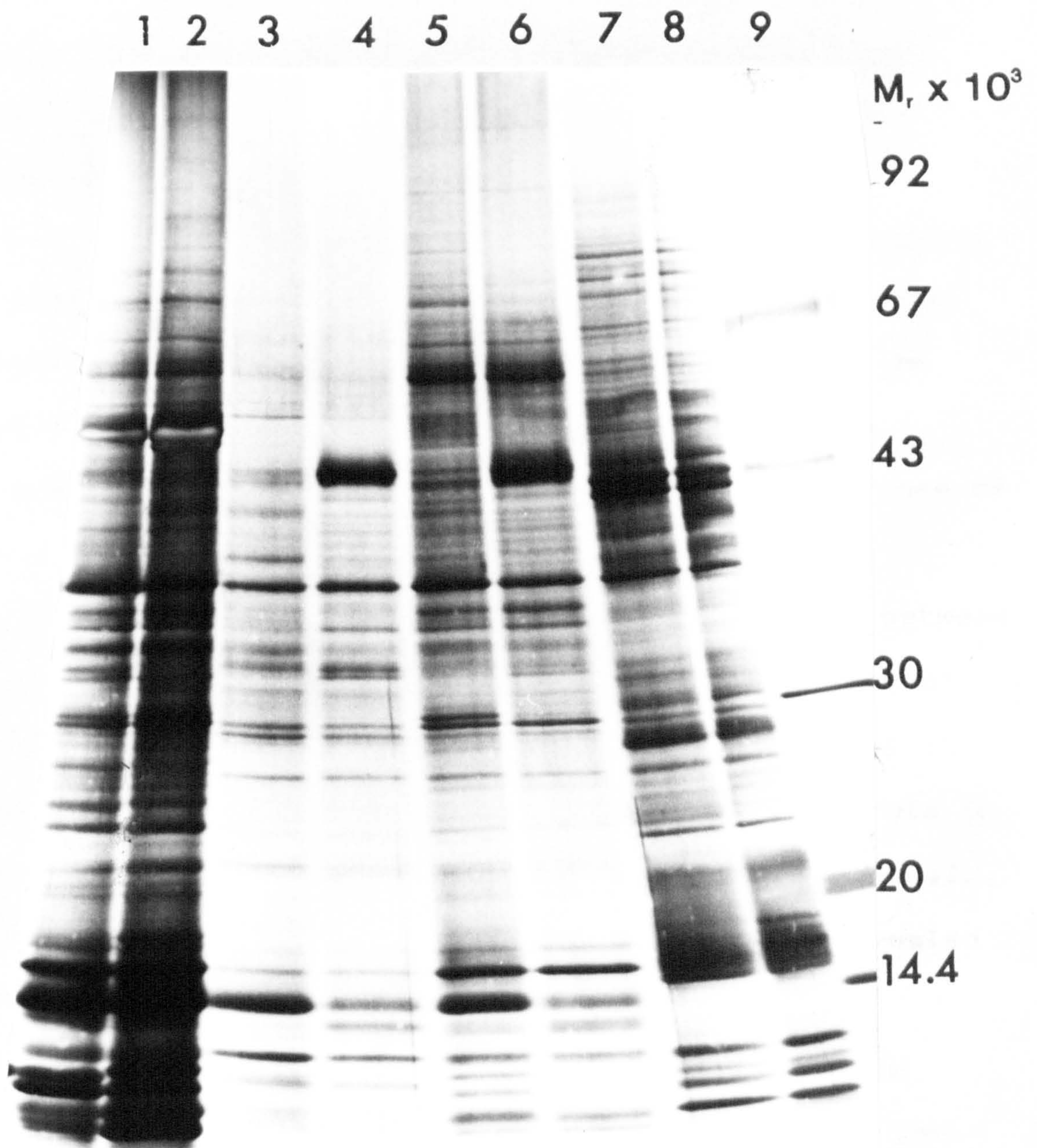
exponential SDS polyacrylamide gel. Figure 4.4 (in which I would like to acknowledge the assistance of D.Scanlan, this laboratory, in running and staining the gel) shows the electrophoretic profile of silver stained polypeptides from the pelleted fraction (1 and 2), cytoplasmic membrane fraction (3 and 4), thylakoid membrane fraction (5 and 6) and the soluble fraction (7 and 8).

No appreciable differences were apparent in the soluble or the pelleted fractions from high and low CO<sub>2</sub>-grown cells. There were however a number of differences in the relative amounts of protein found in the thylakoid and cytoplasmic membrane fractions from the two cultures. The Mr 43,000 polypeptide was present in both the cytoplasmic and thylakoid membrane fractions from low CO<sub>2</sub>-grown cells where it appeared to be the most abundant protein in these fractions. This protein also appeared in high CO<sub>2</sub>-grown cells, however it was present in much lower quantities. Omata and Ogawa (1985 and 1986) reported that in high CO<sub>2</sub>-grown cells of *A. nidulans* R2, the 42 kDa protein accounted for between 3-5% of the total proteins in the cytoplasmic membrane fraction, whilst in low CO<sub>2</sub>-adapted cells this figure increased to 15-25% of the total proteins found in this membrane fraction. Omata and Ogawa (1986) also found the 42 kDa protein in the thylakoid membrane fraction, along with a 37 kDa protein and they considered that the presence of both of these proteins was due to contamination by cytoplasmic membranes, probably from the mixed membrane fraction which bands directly above the thylakoid membrane fraction on these sucrose



Figure 4.4 : SDS-PAGE of *Synechococcus* PCC7942 membrane fractions

Silver stained 10-30% exponential gradient gel, showing membranes isolated from high and low CO<sub>2</sub>-grown *Synechococcus* PCC7942. Tracks 1,3,5 and 7 were from high CO<sub>2</sub>-grown cells, tracks 2,4,6 and 8 were from low CO<sub>2</sub>-grown cells. Tracks 1 and 2, sediment (including cell walls): tracks 3 and 4, cytoplasmic membrane fraction; tracks 5 and 6, thylakoid membrane fraction; tracks 7 and 8, soluble fraction. Track 9, molecular weight markers.





gradients (see Fig. 4.1). Omata and Ogawa (1985 and 1986) reported that there were several smaller changes in the polypeptide composition of the cytoplasmic and thylakoid membrane fractions between high CO<sub>2</sub>-grown and low CO<sub>2</sub>-adapted cells, but none of these were significant changes. A number of changes in the cytoplasmic and thylakoid membrane fractions between high and low CO<sub>2</sub>-grown cells can be seen in Figure 4.4. These included an increase in the amount of polypeptides Mr 52,000 41,000, 39,000 and 16,000 and a decrease in the amount of polypeptides Mr 35,000 and 32,000 in the cytoplasmic membrane fraction from high as opposed to low CO<sub>2</sub>-grown cells. In addition there were a number of polypeptides which were present in greater quantities in the thylakoid membrane fraction from high as opposed to low CO<sub>2</sub>-grown cells, including four of Mr 72,000, 30,000, 11,000 and 10,000 where there were hardly detectable quantities of the protein present in the membranes of low CO<sub>2</sub>-grown cells.

As already stated, although there was a good correlation between the appearance of the 42 kDa polypeptide and the induction of the Ci concentrating mechanism (see Omata and Ogawa, 1986) it has since been proven that this protein plays no direct role in the process of Ci transport in *A. nidulans* (see Schwarz et al., 1988; Omata et al., 1990). However, as figure 4.3 has revealed there are a number of other proteins that vary in amount depending on the growth condition. These differences could however simply represent the normal fluctuations seen in the amounts of various proteins during the growth cycle of the



cells, since although attempts were made to harvest cultures at the same point in the growth cycle, the cultures had different growth rates due to the different CO<sub>2</sub> regimes and so differences in the amounts of the various cellular components are likely.

#### 4.3.4 SDS-PAGE of membrane fractions isolated from *Synechocystis* PCC6803

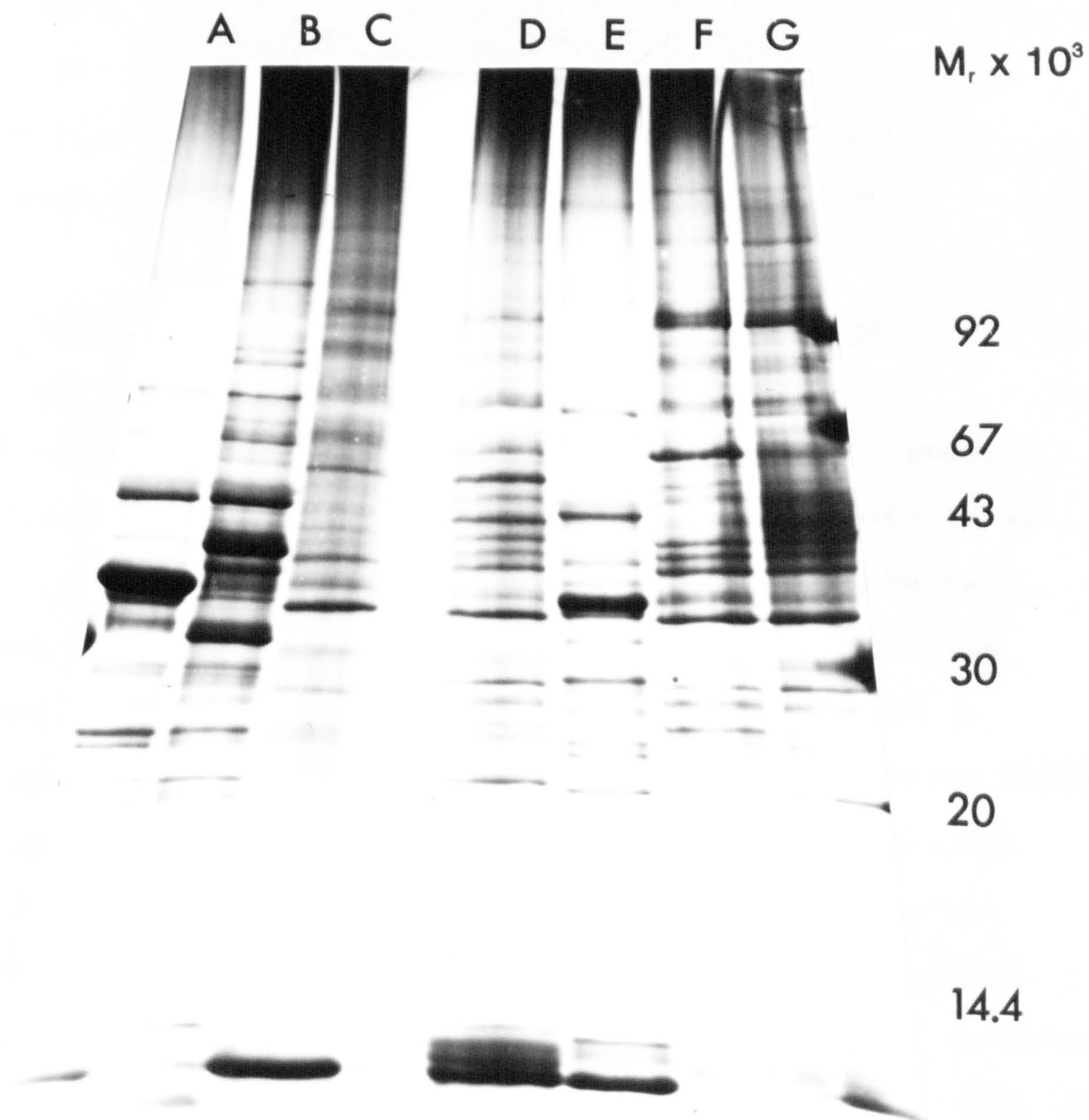
Figure 4.5 shows the polypeptide profile following SDS-PAGE of approximately equal amounts of protein (20ug) from thylakoid and cytoplasmic membrane fractions isolated from *Synechocystis* PCC6803 grown under heterotrophic and high and low CO<sub>2</sub> regimes. Unlike *Synechococcus* PCC7942 there was little difference in the polypeptide profile of the cytoplasmic membrane fractions of cells grown under high and low CO<sub>2</sub> conditions, a similar finding to that made by Omata and Ogawa (1987). There were differences however between the cytoplasmic membrane profile of cells grown under these two conditions and those grown heterotrophically. In particular a polypeptide of Mr 35,000 was found in much greater abundance in the cytoplasmic membrane fraction from high and low CO<sub>2</sub> grown than in heterotrophically grown cells and conversely, a polypeptide Mr 30,000 was found in much greater abundance in the cytoplasmic membrane fraction from heterotrophically grown cells. However, apart from these differences, the polypeptide profiles were remarkably similar. There were other subtle differences which could be significant, genuine differences, in protein levels between growth regimes.

The polypeptide profiles of the thylakoid membrane fractions



Figure 4.5 : SDS-PAGE of *Synechocystis* PCC6803 thylakoid and cytoplasmic membrane fractions.

Silver stained 10-30% exponential gradient gel, showing cytoplasmic (cyto) and thylakoid (thy) membrane fractions isolated from heterotrophic (Het) and high (H) and low (L) CO<sub>2</sub>-grown cultures of *Synechocystis* PCC6803. Track A, 6803L cyto; track B, 6803Het cyto; track C, 6803L thy; track D, 6803Het thy; track E, 6803H cyto; track F, 6803H thy; track G, 6803H thy (from stationary phase culture)





were also remarkably similar under all growth conditions. In fact there was less variation in the composition of the thylakoid membranes than there was in the cytoplasmic membranes. The fact that many of the metabolic pathways involved are common must be a contributing factor to the similarity of the polypeptide profiles, however, considering the different substrate acquisition and energy requirements that each of these growth conditions poses to the cell, it suggests that control of these processes probably occurs, at least to an extent, through protein modifications, although it is known that the sugar transporting mechanism of *Synechocystis* PCC6803 uses non-phosphoryllating processes (Zhang et al., 1989).

#### 4.3.5 Growth of *Synechococcus* PCC7942 in light and $\text{CO}_2$ -limited chemostat cultures

Unlike Miller et al. (1984a), whose approach was to study *S. leopoliensis* in chemostat culture at different growth rates using a standard  $\text{Ci}$  concentration (10 mM  $\text{NaHCO}_3$ ), the main approach taken in this study, was to keep the growth rate constant and vary the DIC concentration. At some DIC concentrations however the growth rates of *Synechococcus* PCC7942 were varied to enable  $\mu_{\text{max}}$  to be calculated (see Fig 4.7). Early experiments concentrated on growth of *Synechococcus* PCC7942 in  $\text{NaHCO}_3$ -limited chemostats. However, it proved very difficult to maintain steady-state cultures due to i) persistent wall growth which occurred in most cultures after 4-5 days and ii) the inability of *Synechococcus* PCC7942 to grow in chemostat



cultures when the  $\text{NaHCO}_3$  concentration was lower than 5 mM. The chemostat cultures used by Karagouni and Slater (1979) were maintained for over two years (per. comm.), and as chemostat cultures provide very high selection pressures for microorganisms (see review by Harder et al., 1977) it may be that over this period of time they selected for a cyanobacterial strain that could grow very well at low bicarbonate concentrations. Due to this problem, it was decided to carry out all further work in chemostat cultures gassed with varying percentages of  $\text{CO}_2$ : $\text{CO}_2$ -free air (or  $\text{N}_2$ ) and air: $\text{CO}_2$ -free air. The pH in all of these experiments was kept constant (pH 8.2) as described in section 2.8. It was found that unless the concentration of acid and base used with the pH controller were kept reasonably low (0.1M) in these chemostat systems, it was very difficult to maintain a steady-state, as the pH fluctuated wildly, with subsequent fluctuations in the biomass. This is probably due to the low "inherent" buffering ability of the growth medium when it does not contain any  $\text{NaHCO}_3$ . When the pH controller was switched off, the pH in the culture vessel rose to around 9.3, followed by an increase in the culture biomass. This increase in pH is, given the constancy of alkalinity during  $\text{CO}_2$  exchanges, probably due to the depletion of  $\text{CO}_2$  during algal photosynthesis, which was shown as early as 1926 (Schutow, 1926, cited by Talling, 1985) to drive pH up to high values. The increase in biomass can be explained in terms of the solubility of  $\text{CO}_2$  in solution. At pH 9.3, the total DIC concentration in the culture vessel will increase. Above pH 7.0

Figure 4.6 : Steady-state growth in chemostat  
cultures of *Synechococcus* PCC7942

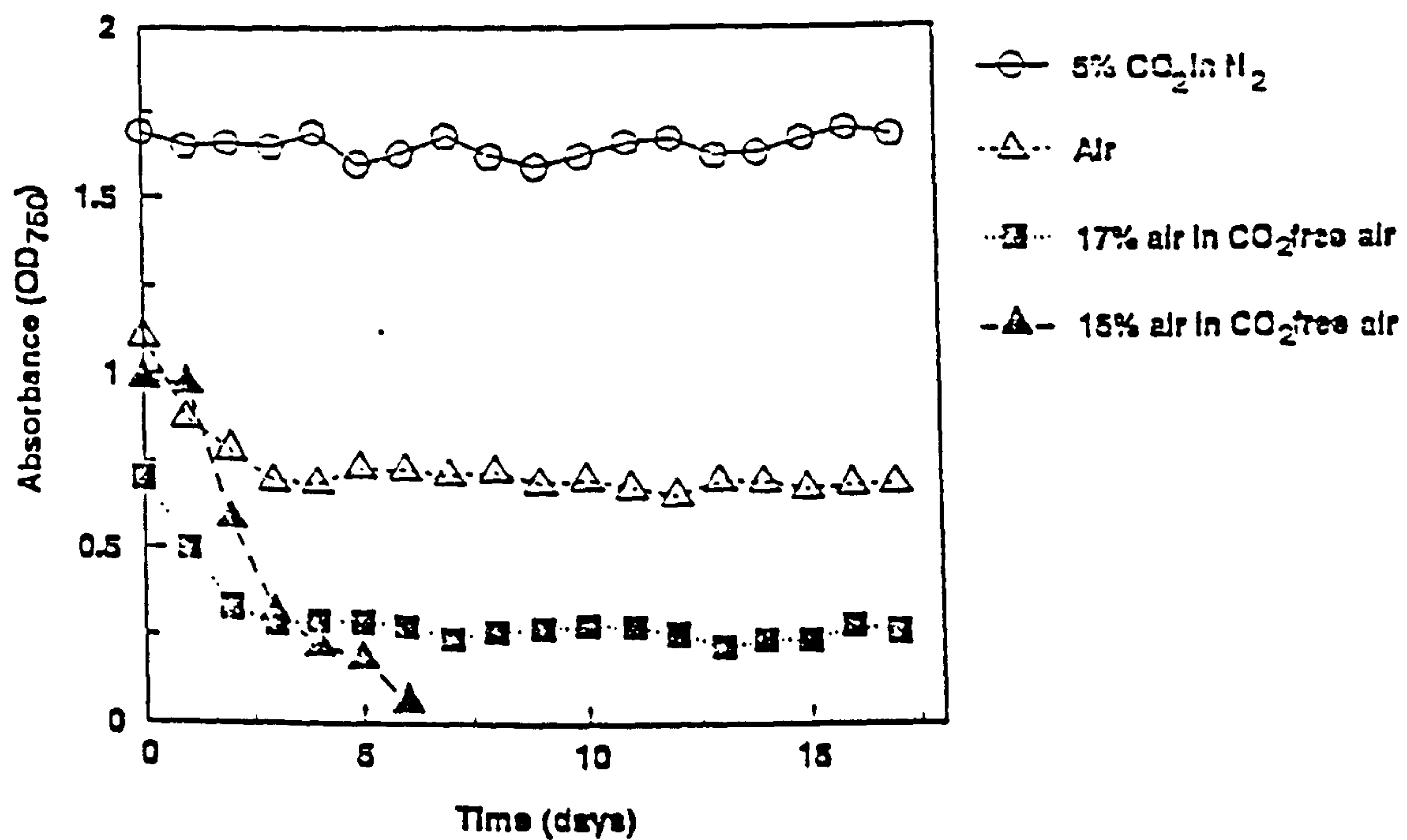
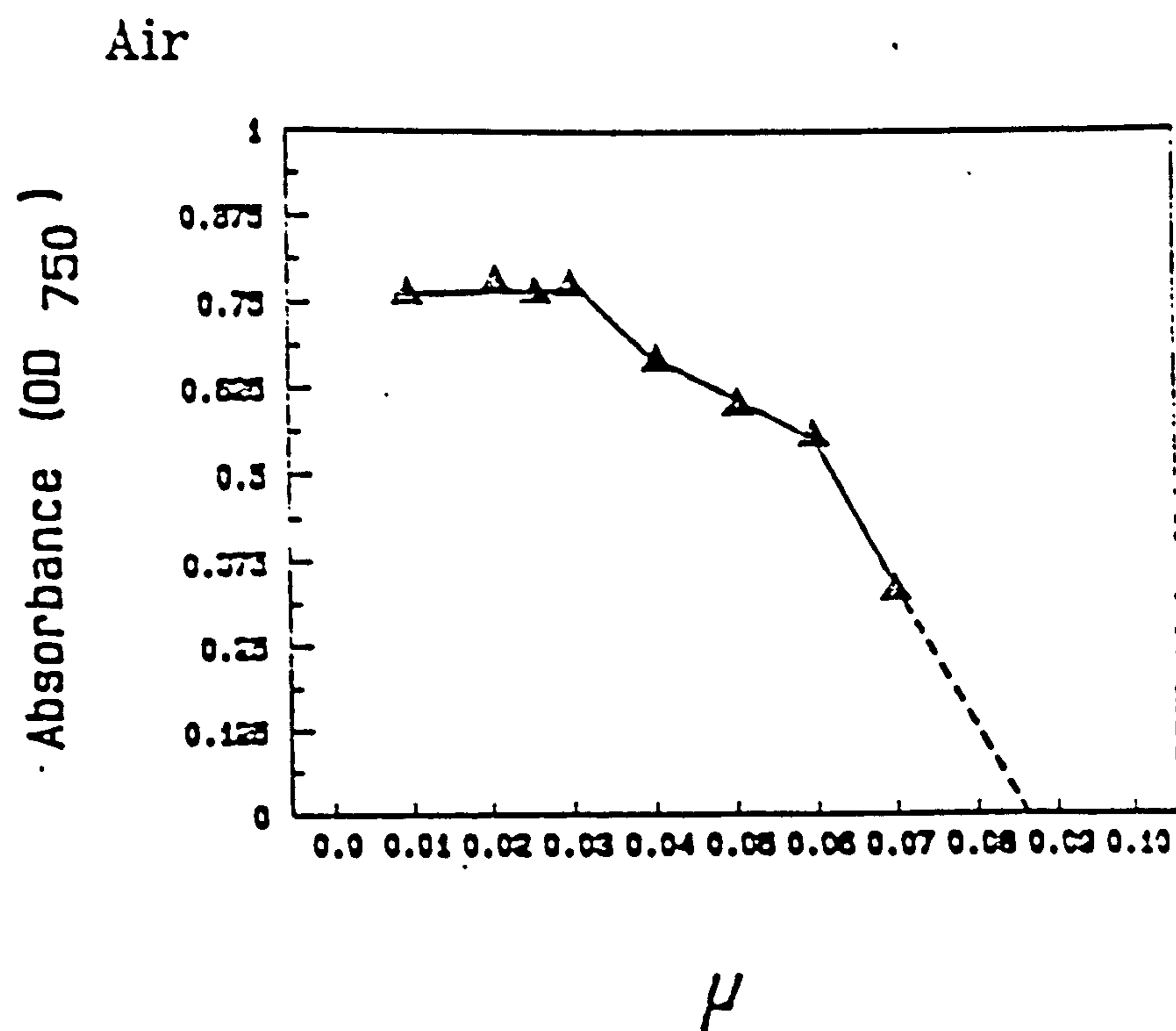
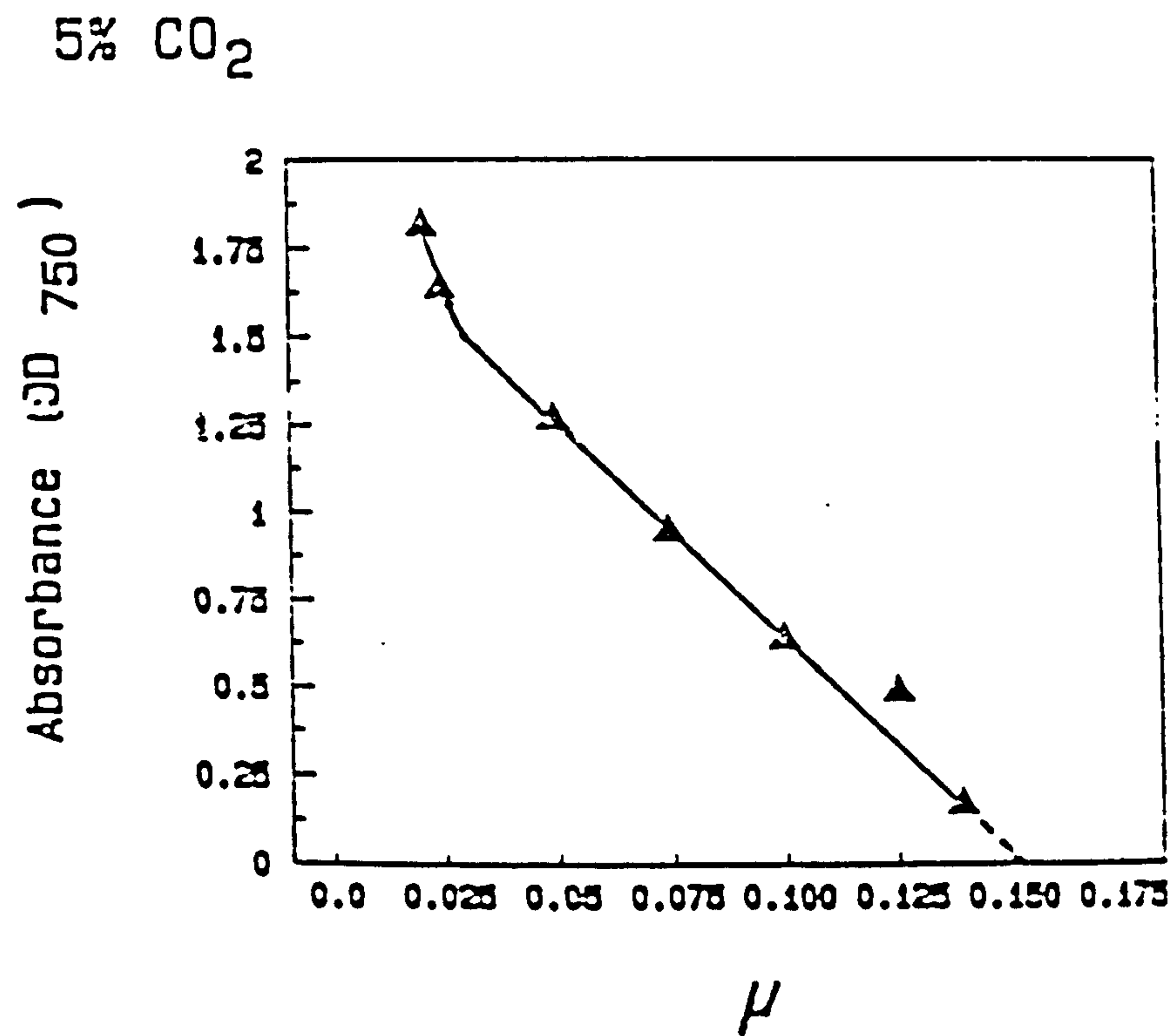


Figure 4.7 : Effect of increasing dilution rate on chemostat  
cultures of *Synechococcus* PCC7942





there is a 10-fold increase in  $C_i$  for each 1 unit rise in pH (Miller, 1990) and as it has been well established that in cyanobacteria there is mediated transport for both  $\text{CO}_2$  and  $\text{HCO}_3^-$  (see Miller et al., 1990), greater concentrations of organisms can be supported in the chemostat at this elevated pH.

Figure 4.6 shows the typical  $\text{OD}_{750}$  results of steady-state growth of *Synechococcus* PCC7942, grown at a dilution rate of  $0.023 \text{ h}^{-1}$  (td 30 hr) over a range of different DIC concentrations. Similar results were obtained with dry weight measurements and chlorophyll determinations. It can be seen that as the amount of  $\text{CO}_2$  supplied to the culture is reduced, there is a corresponding drop in the culture biomass, until a point is reached where the substrate concentration supplied cannot sustain growth at the set dilution rate. This critical substrate concentration ( $S_{\text{crit}}$ ) was reached when the air: $\text{CO}_2$ -free air ratio fell below 17%, at a dilution rate of  $0.023 \text{ h}^{-1}$ . Below this  $S_{\text{crit}}$  culture washout occurred, as can be seen from the 15% air in  $\text{CO}_2$ -free air plot in Fig. 4.6.

Varying the growth rates of chemostat cultures grown under  $\text{CO}_2$  and light limited conditions (see Fig. 4.7) produced the same nutrient-limited chemostat pattern as seen by Karagouni and Slater (1979). Under light-limited conditions the biomass continued to increase significantly with decreasing dilution rate, and supported much higher growth rates than  $\text{CO}_2$ -limited cultures. By extrapolation of the graphs, a  $u_{\text{max}}$  of  $0.15 \text{ h}^{-1}$  (td 4.62 hr) was obtained for the light-limited chemostat culture, and one of  $0.08 \text{ h}^{-1}$  (td 8.66 hr) for the  $\text{CO}_2$ -limited culture.

In theory culture washout should occur when the dilution rate is increased above these figures. In reality it was found that the  $\text{CO}_2$ -limited chemostat followed the model very closely, as above dilution rates of  $0.08 \text{ h}^{-1}$  culture washout occurred. However, in the light-limited chemostat it was very difficult to maintain steady-state cultures above a dilution rate of  $0.130 \text{ h}^{-1}$  (td 5.33 hr), and above  $0.140 \text{ h}^{-1}$  (td 4.95 hr) culture washout always occurred.

Karagouni and Slater (1978) studied growth of *Anacystis nidulans* during washout from light and  $\text{CO}_2$ -limited chemostats to determine  $u_{\text{max}}$  and found that culture washout (especially in the initial stages) was not a reliable technique for determining  $u_{\text{max}}$  values. They suggested this was because when the dilution rate increased, the concentration of the growth-limiting nutrient also had to rise to levels which did not restrict the populations growth rate and it was not always possible to ensure this, and on a more fundamental physiological level that the lag seen in classical "shift-up" experiments with heterotrophic cultures may also be operating under these conditions.

The reason why the theoretical and experimental  $u_{\text{max}}$  values determined in these experiments are reasonably close is probably because the dilution rate was increased in a stepwise fashion very slowly, with the culture being allowed to establish a steady-state before the dilution rate was increased, unlike Karagouni and Slater (1978), who simply switched from a low to a high dilution rate. Miller et al. (1984a), using a similar technique to that adopted here, also found good correlation

between theoretical and experimental values, although they did compare values obtained in the chemostat to those in batch culture. Considering the high selection pressure applied in chemostat cultures (see Harder et al., 1977), and the often different growth conditions and growth rates employed, this would not appear to be a good idea.

#### 4.3.6 DIC uptake and concentration by *Synechococcus* PCC7942 in chemostat cultures

The concentration of the three main inorganic carbon species found in solution at pH 8.2, at each of the different culture conditions employed in this study is shown in Table 4.4.

The solubility of  $\text{CO}_2$  in a liquid, is in essence no different from that of any other gas, being dependent on several factors such as temperature, pressure and the presence of salts.

However, unlike most other gases, the carbon dioxide dissolved in solution participates in interconnected equilibria (see Figure 1.5) that typically involve much larger quantities of the gas bound in ionic form as bicarbonate, or at high pH, carbonate ions.

A useful term employed to express the solubility of a gas in a liquid, at a fixed temperature and pressure is the Bunsen absorption coefficient, which is defined as "that volume of gas in litres at STP which saturates 1 litre of liquid when it is presented to the liquid at the reported temperature and a partial pressure of 1 atmosphere". At atmospheric pressure, the solubility of  $\text{CO}_2$ , like most other gases is inversely



proportional to the temperature. A series of solubility constants have been derived for a number of gases at a given temperature and a partial pressure of 1 atmosphere, and the one used in this study, 0.665, is that from the Handbook of Chemistry and Physics (1963), and cited by Umbreit (1964). This review states that "the presence of salts, etc., in solution has little effect upon the solubility of carbon dioxide, within physiological concentrations" and so for the purpose of the calculations employed in this study, it is assumed that the solubility of  $\text{CO}_2$  in BG11 is the same as that in pure water. Hence to work out the concentration of free  $\text{CO}_2$  in BG11 at  $30^\circ\text{C}$ , the atmospheric concentration of  $\text{CO}_2$  (taken as 0.00034 atm) is simply multiplied by the solubility constant.

There are a number of ways of calculating the total DIC. It can be determined unequivocally by acidifying the sample beyond the bicarbonate end-point, removing the gaseous  $\text{CO}_2$  in a stream of carrier gas and then determining the  $\text{CO}_2$  by physical methods (i.e. infra-red gas analysis or gas chromatography).

Alternatively, the  $\text{CO}_2$  produced can be absorbed in dilute alkali and then estimated by titration with mineral acid or more conveniently from the change of conductivity of the alkali solution. However, the method chosen for determining the concentration of the other inorganic carbon species present in solution at pH 8.2 in this study, relies on indirect calculations based upon dissociation constants applicable to the carbon dioxide-bicarbonate-carbonate buffer system, basically derivations of the Henderson-Hasselbach equation, shown overleaf

$$\text{pH} = \text{pK}_a + \log \frac{[\text{conjugate base}]}{[\text{acid}]} \quad 4.4$$

where  $\text{pK}_a$  is the dissociation constant of the acid.

As  $\text{pH} = -\log [\text{H}^+]$ , this can be substituted to give for bicarbonate :-

$$[\text{HCO}_3^-] = \frac{\text{pK}_a \times [\text{CO}_2]}{[\text{H}^+]} \quad 4.5$$

where  $\text{pK}_a$ , the first apparent dissociation constant of carbonic acid = 6.33 [ $K_1 = 4.68 \times 10^{-7}$ ] (Lyman, 1956, cited by Stumm and Morgan, 1981).

and for carbonate :-

$$[\text{CO}_3^{2-}] = \frac{\text{pK}_a \times [\text{HCO}_3^-]}{[\text{H}^+]} \quad 4.6$$

where  $\text{pK}_a$ , the second apparent dissociation constant of carbonic acid = 10.29 [ $K_2 = 5.12 \times 10^{-11}$ ] (Lyman, 1956, cited by Stumm and Morgan, 1981).

One other qualifying point that must be made is that the figures given for DIC concentrations in Table 4.4 make no calculation for the Ci being depleted from the culture because of cell growth, however it is assumed that because there is a constant influx of  $\text{CO}_2$ , that at a given dilution rate the organism establishes a steady-state, and external DIC remains constant.

As can be seen from Table 4.4, the chemostat culture conditions used resulted in total DIC concentrations ranging from 110.3 mM down to 0.128 mM. Under these conditions the RuBisCO activity increased from 4 to 25 nmol CO<sub>2</sub> fixed min per mg dry weight, a 6 fold change in activity. Karagouni and Slater (1979) reported a 15-fold increase in the activity of RuBisCO with decreasing dilution rate in CO<sub>2</sub>-limited cultures. This increase did not occur stepwise, with a continuum of RuBisCO activities as total external DIC concentration fell, but rather, there were three distinct stages, where RuBisCO activity increased markedly over the preceeding DIC concentration. The lowest RuBisCO activities were encountered in cultures gassed with a mix of CO<sub>2</sub>:N<sub>2</sub>, where the total DIC varied between 6.64 and 110 mM. In these cultures RuBisCO activity ranged between 4 and 5.8 nmol CO<sub>2</sub> fixed per mg dry weight. The next distinct stage was represented by cultures gassed with air or air:CO<sub>2</sub> free air mixtures where the DIC concentration ranged from 0.756 mM down to 0.296 mM. In these cultures RuBisCO activity ranged between 12 and 16 nmol CO<sub>2</sub> fixed per mg dry weight. In chemostat cultures with DIC below 0.153 mM, down to a minimum of 0.128 mM, where any further reduction in the DIC concentration caused chemostat washout, RuBisCO activity was between 23.5 and 25 nmol CO<sub>2</sub> fixed per mg dry weight. This discontinuous alteration in RuBisCO activity as DIC concentration and biomass decrease suggests that some kind of enzyme activation or stabilization is occurring, as opposed to some kind of transcriptional activation.



Table 4.4 : DIC concentrations and RuBisCO activity in chemostat cultures of *Synechococcus* PCC7942

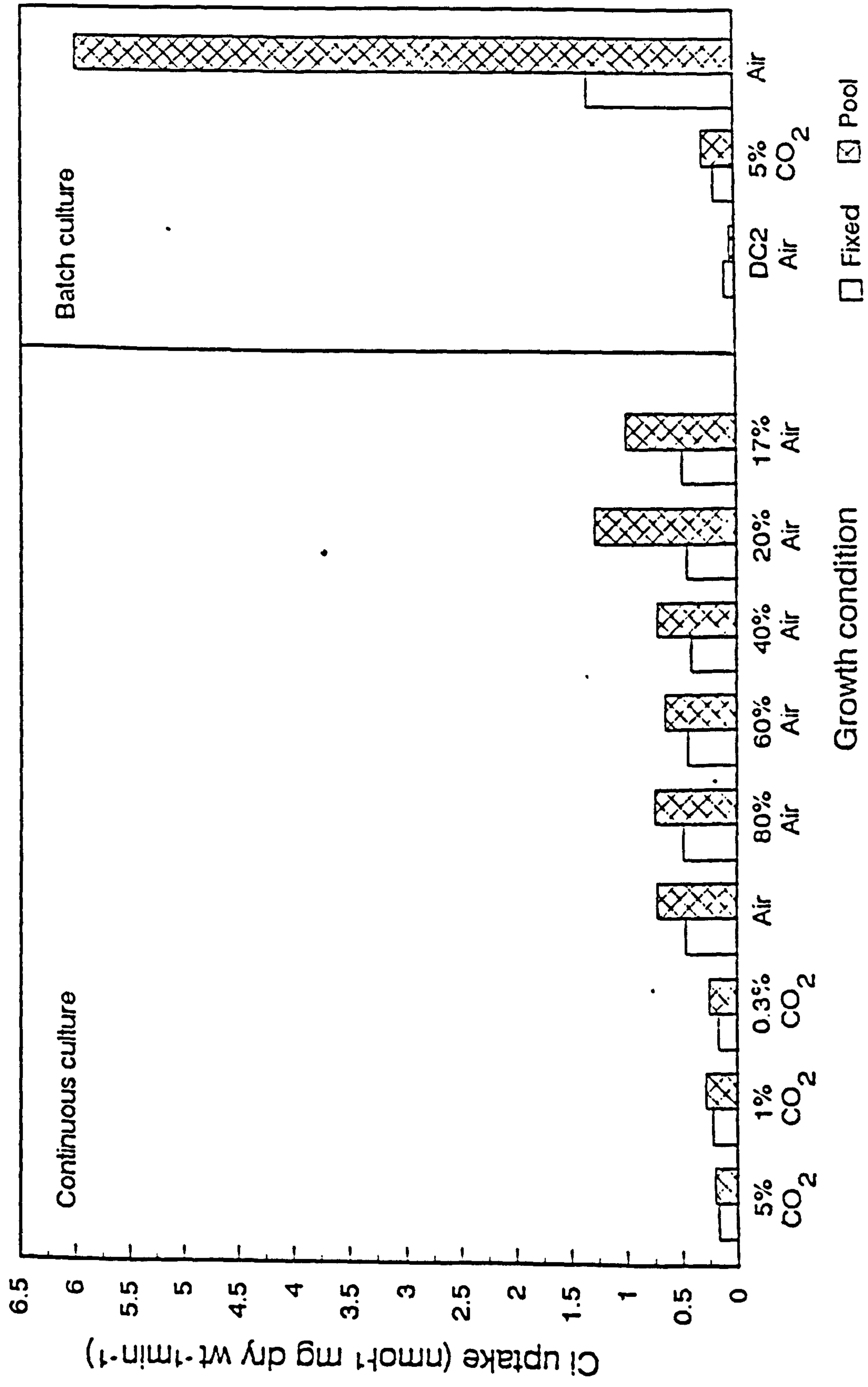
Culture conditions	DIC concentration			RuBisCO activity nmol CO <sub>2</sub> min /mg dry wt
	CO <sub>2</sub>	HCO <sub>3</sub> <sup>-</sup>	CO <sub>3</sub> <sup>2-</sup>	
5% CO <sub>2</sub>	1.46 mM	108 mM	0.88 mM	4.0
1% CO <sub>2</sub>	0.29 mM	22 mM	0.18 mM	5.8
0.3% CO <sub>2</sub>	87 μM	6.5 mM	53 μM	4.3
100% Air	9.95 μM	0.74 mM	5.99 μM	12.0
80% Air	7.96 μM	0.59 mM	4.79 μM	13.5
60% Air	5.97 μM	0.42 mM	3.59 μM	14.0
40% Air	3.98 μM	0.29 mM	2.39 μM	16.0
20% Air	1.99 μM	0.15 mM	1.20 μM	23.5
17% Air	1.69 μM	0.125 mM	1.02 μM	25.0

Figure 4.8 shows the characteristics of DIC uptake and fixation under the same chemostat conditions as those shown in Table 4.4. The most striking feature of the results, which is discussed later in this chapter, is the very low internal DIC levels maintained by *Synechococcus* PCC7942 in chemostat culture. Comparing Table 4.4 with Figure 4.8 it can be seen that *in vitro* RuBisCo activities are always greater than the DIC saturated rate of photosynthesis calculated from vacuum filtration experiments, which Mayo et al. (1989) have found is consistent with the rate of RUBP regeneration setting the maximum rate of photosynthesis *in vivo*.

In batch cultures grown at high external DIC concentrations, reported levels of RuBisCO and RUBP and *in vitro* RuBisCO activity are higher than those grown at low external DIC concentrations (see Mayo et al., 1989 and Table 3.1) and as less energy needs to be diverted to maintain the highly energy dependent  $C_i$ -concentrating mechanism (see Raven and Lucas, 1985) more  $CO_2$  is fixed by high  $CO_2$ -grown cells. Those chemostat cultures gassed with higher levels of  $CO_2$  did not have higher *in vitro* RuBisCO activities, in fact the reverse was true, however they did support greater culture biomass. This was probably simply due to the "carrying capacity" of the medium, the excess DIC allowed far more organisms to be supported, rather than being due to any different physiological characteristics these cells possess.

The term  $C_i$ -limitation is used here cautiously however, as although the cells grown under air or air: $CO_2$ -free air mixtures

Figure 4.8 :  $\text{Ci}$  uptake in chemostat cultures of *Synechococcus* PCC7942





were in chemostat terms  $\text{CO}_2$ -limited for growth, with an increase in culture biomass accompanying any increase in added  $\text{CO}_2$  (and therefore DIC) from the results shown in Figure 4.8 it would seem that the chemostat-cultures employed in this study were not actually Ci-limited with respect to the Ci-concentrating mechanism.

Although it has earlier been stated that direct comparisons with batch cultures should be treated carefully because of the tremendous selection pressure placed upon organisms in chemostat cultures and the often different growth rates employed, it can be seen from the results in Figure 4.8 that both Ci uptake into the internal Ci pool and Ci fixation into acid-stable products are far below figures seen in low  $\text{CO}_2$ -grown batch cultures of the same organism. The uptake rates, in fact far more resemble the figures for high  $\text{CO}_2$ -grown batch cultures. Only in those cultures gassed with 20 and 17% air are the internal Ci pools higher than in high  $\text{CO}_2$ -grown batch cultures. In addition, at an assay time of 30 seconds for organisms grown in DIC-limited batch culture, the internal Ci pool, in general represents between 70 and 85% of the total Ci taken up by the cells. However, from the results of this chemostat culture study it can be seen that the internal pool generally only represents roughly 60% of the DIC transported into the cell. Also included in Figure 4.8 for comparison, are Ci uptake rates obtained from low  $\text{CO}_2$ -grown *Synechococcus* WH7803 (DC2). Although the rates of Ci uptake in chemostat cultures of *Synechococcus* PCC7942 are lower than we might have expected, given the low external DIC

concentrations, they are nowhere near as low as those seen in *Synechococcus* WH7803, which has already been shown to lack a  $\text{Ci}$  uptake mechanism under the conditions employed (see Fig. 3.6 and 3.7). Under the conditions employed in the chemostat, similar growth rates were obtained to those seen in *Synechococcus* WH7803 (see Fig. 3.3), so it would seem that growth rate alone is not responsible for the apparent absence of a  $\text{Ci}$  uptake mechanism in this species, since there was a low rate of  $\text{Ci}$  uptake in these chemostat cultures..

There have been a number of reports on photosynthetic adaptation in response to growth at different DIC concentrations in batch culture grown *Synechococcus* PCC6301, or *S. leopoliensis* UTEX 625 as it is also known (see Mayo et al., 1986 & 1989; Badger and Gallacher, 1987). These authors have suggested that it is not  $\text{CO}_2$  concentration *per se*, but the DIC concentration at which the cells are grown that determine the photosynthetic responses of the cell, a similar finding to that made by Miller et al.

(1984a) in their continuous-culture studies. Of great significance to this work is the fact that these authors have reported that there are certain external DIC concentrations above which the cells are repressed in terms of bicarbonate transport (above 1.5-2 mM), and below which the cells become fully adapted for bicarbonate transport, being truly DIC-limited. Badger and Gallacher (1987) found the DIC concentration below which the cells became fully DIC-limited to be 50  $\mu\text{M}$ , whilst Mayo et al. (1986) reported that only at DIC concentrations below 20-40  $\mu\text{M}$  would growth become DIC-limited.

Miller et al. (1984a) working in chemostat cultures at pH 9.2 found that "low  $\text{Ci}$ -adapted cells" were produced if growth DIC was below 0.1 mM and that "high  $\text{Ci}$  adapted cells" were produced when growth DIC was above 2 mM. None of the DIC concentrations used in this study reach these levels, which probably explains why  $\text{Ci}$  uptake into the internal pool occurs at much lower levels than seen in batch culture. The DIC concentration in the medium where culture washout occurred was 0.11 mM, and it may be that at the slow growth rates used in this series of experiments the cells never sensed themselves as being DIC-limited for growth and so did not induce the  $\text{Ci}$ -concentrating mechanism. However, under these conditions of substrate concentration and dilution rate they were probably, using the data of Mayo et al. (1986) as a guideline, approaching the point where the  $\text{Ci}$  concentrating mechanism needed to become fully induced, and because it was not induced,  $S_{\text{crit}}$  for the uninduced cells was reached, and the culture washed out.

The range of DIC concentrations actually used in this series of experiments represent in chemostat culture terms, if we take the figures of Miller et al. (1984a), the intermediate DIC concentrations between being fully repressed and fully induced in terms of active  $\text{Ci}$  transport. According to Mayo et al. (1986 & 1989) and Badger and Gallacher, (1987) a continuum of cell types with intermediate affinities for  $\text{Ci}$  transport should be found under such conditions. Although there were differences in the RuBisCO activities, with three different "groups" of activity being found, there was no real continuum of cells types



with regards  $C_i$  uptake into the internal pool in chemostat culture. Admittedly those cells from cultures with DIC levels above 6.5 mM had lower internal  $C_i$  pools than cells from the remaining cultures, but the pool sizes in cells from the remaining cultures were very similar, and were only 2-3 times higher than the lowest pool sizes found. This lack of a continuum of cells types with differing responses to external DIC is not surprising since other workers using chemostat cultures have shown a sharp transition in the affinity of the cells for DIC, and predicted that only two cell types can be produced, cells that are fully induced or fully repressed for  $C_i$  uptake, with any intermediate characteristics in chemostat cultures due to a mixture of these cell types (see Miller et al., 1984a; Turpin et al., 1985b; Mayo et al., 1986). The higher  $C_i$  pool sizes, and increased RuBisCO activity seen in cultures gassed with 20 and 17% air may reflect this fact, with a small proportion of the population of cells becoming fully induced with respect to  $C_i$  uptake and RuBisCO activity. The chemostat culture would still washout if too small a percentage of the population became fully induced because obviously each cell has a finite residence time in the culture. Alternatively, the increase in  $CO_2$ -fixed in those cultures gassed with 20 and 17% air in  $CO_2$ -free air may simply reflect the increased activity of RuBisCo the organisms under these growth conditions display, but this would not account for the increased  $C_i$  pools maintained by the cells under these conditions.

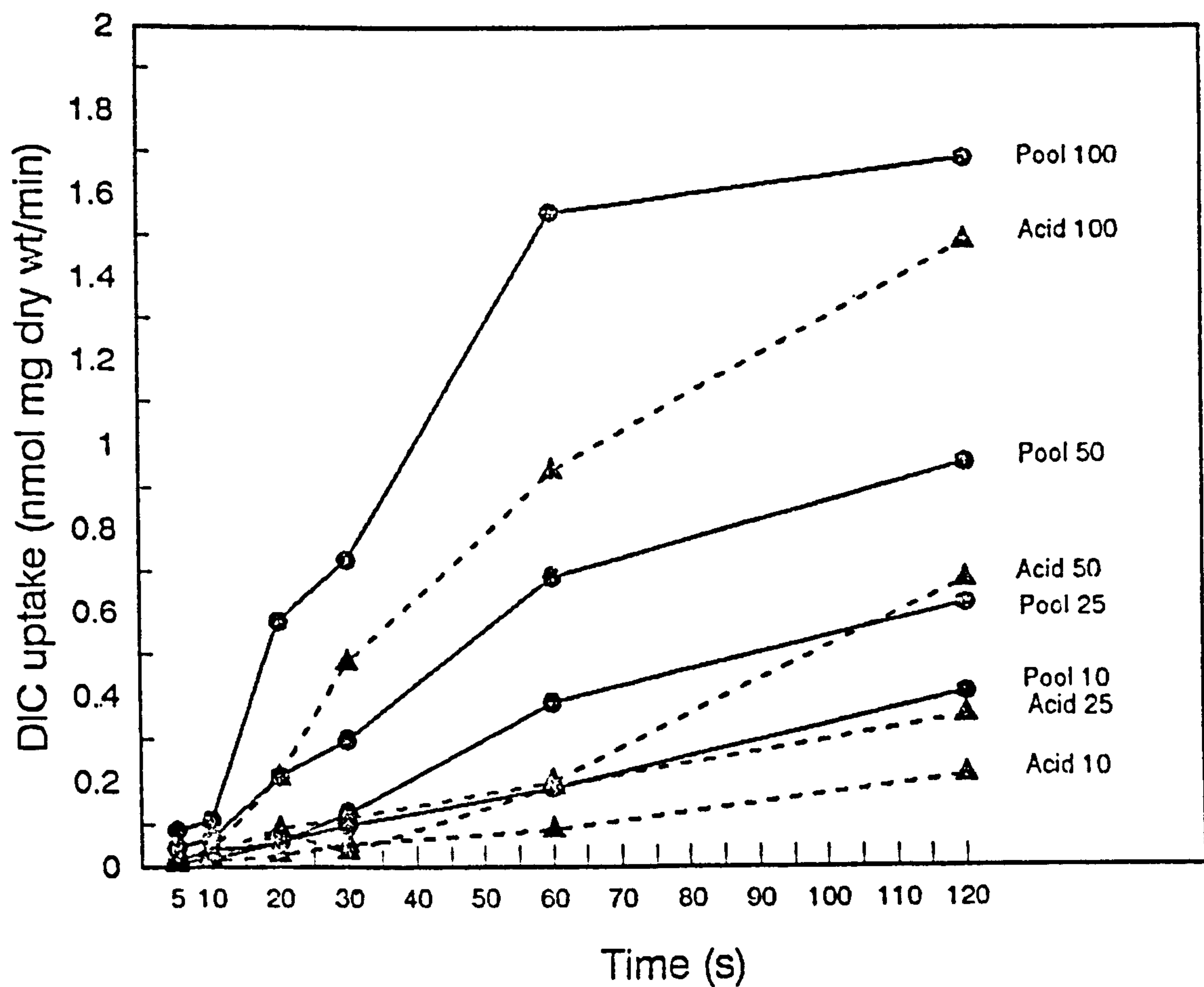
This lack of intermediate values has been explained by Turpin et

al. (1985b) as being due to the nature of steady-state chemostat culture, where the cyanobacteria must maintain a growth rate equivalent to the culture dilution rate and must therefore establish an exogenous DIC concentration accordingly. Hence for long term stability to be maintained in chemostats the affinity of the cells for DIC must be fully induced or fully repressed. Obviously, in these experiments a DIC concentration was not reached at which the cells became fully induced. As washout occurred when the DIC concentration in the chemostat fell to levels below 0.128 mM, it would seem that in the chemostat under the conditions employed this is the lowest DIC concentration which can support growth by cells with repressed Ci uptake mechanisms.

Figure 4.9 shows the time course of Ci uptake by chemostat cultures of *Synechococcus* PCC7942 gassed with air. Under these conditions it has already been established that there is no appreciable Ci concentrating mechanism (see Fig. 4.8). Ci uptake was determined over a range of external DIC concentrations, ranging from 10 - 100  $\mu$ M. A predictable pattern emerged, with the Ci uptake rate increasing with increasing external DIC concentration and time. However, because of the very limited Ci-concentrating mechanism these cells possess, there was no rapid uptake of Ci into the internal pool, as seen in low  $\text{CO}_2$ -grown batch cultures, and only in the experiment with an external DIC concentration of 100  $\mu$ M did the internal Ci pool reach near saturating levels. With the exception of the 100  $\mu$ M time course, where at 120s,  $\text{CO}_2$  fixed into acid insoluble cell

Figure 4.9 : Time course of  $\text{Ci}$  accumulation in air bubbled chemostat cultures of *Synechococcus* PCC7942

Time course showing the accumulation of  $\text{Ci}$  into the internal pool (●) and acid-stable photosynthetic products (▲) at external DIC concentrations ranging from 10 - 100  $\mu\text{M}$  in *Synechococcus* PCC7942





products rose to levels similar to the  $C_i$  transported into the internal  $C_i$  pool, in every other time course,  $CO_2$  fixed into acid-stable products never reached the levels reached in the internal  $C_i$  pool. This suggests that, with the exception of the 100  $\mu M$  time course, at no point in these experiments was  $CO_2$  saturating for RuBisCO, and that because of a combination of a low affinity uptake system together with cells with a slow growth rate that a much longer time course would have to be used to enable the internal  $C_i$  pool to rise to a high enough level to saturate RuBisCO. It must be noted however, that all of the DIC concentrations used were below the steady-state DIC concentrations used in the chemostats in this study, and that at the external DIC of 100  $\mu M$ , which is similar to that seen in the chemostat gassed with 17% air, the internal pool did become saturating. The fact that the size of the internal pool is much lower than that seen in batch cultures may be compensated for in cells grown in continuous culture at DIC concentrations limiting for growth by the increased RuBisCO activities seen in these cells compared to those in which DIC was in excess.

In an attempt to establish  $K_m[DIC]$  and  $V_{max}$  in the chemostat cultures used in this study, experiments were performed where  $C_i$  uptake was measured over a series of external DIC concentrations at  $T=30s$ .

For cultures in which DIC was in excess for growth (light-limited),  $V_{max}$  was  $1.64 \pm 0.054$  nmol mg dry wt  $min^{-1}$  and  $K_m$   $59 \pm 10$   $\mu M$ , whilst in those cultures where DIC was limiting

for growth  $V_{max}$  was  $5.74 \pm 0.246$  nmol mg dry wt min<sup>-1</sup> and  $K_m$   $80 \pm 12$   $\mu$ M.

It can be seen from the results that the affinity of the uptake system for DIC was similar in chemostats in which DIC was in excess or limiting, whilst  $V_{max}$  was three fold greater in DIC limited chemostats. This could help to explain the differences seen in Ci uptake between those chemostat cultures where DIC was limiting over those where it was in excess.

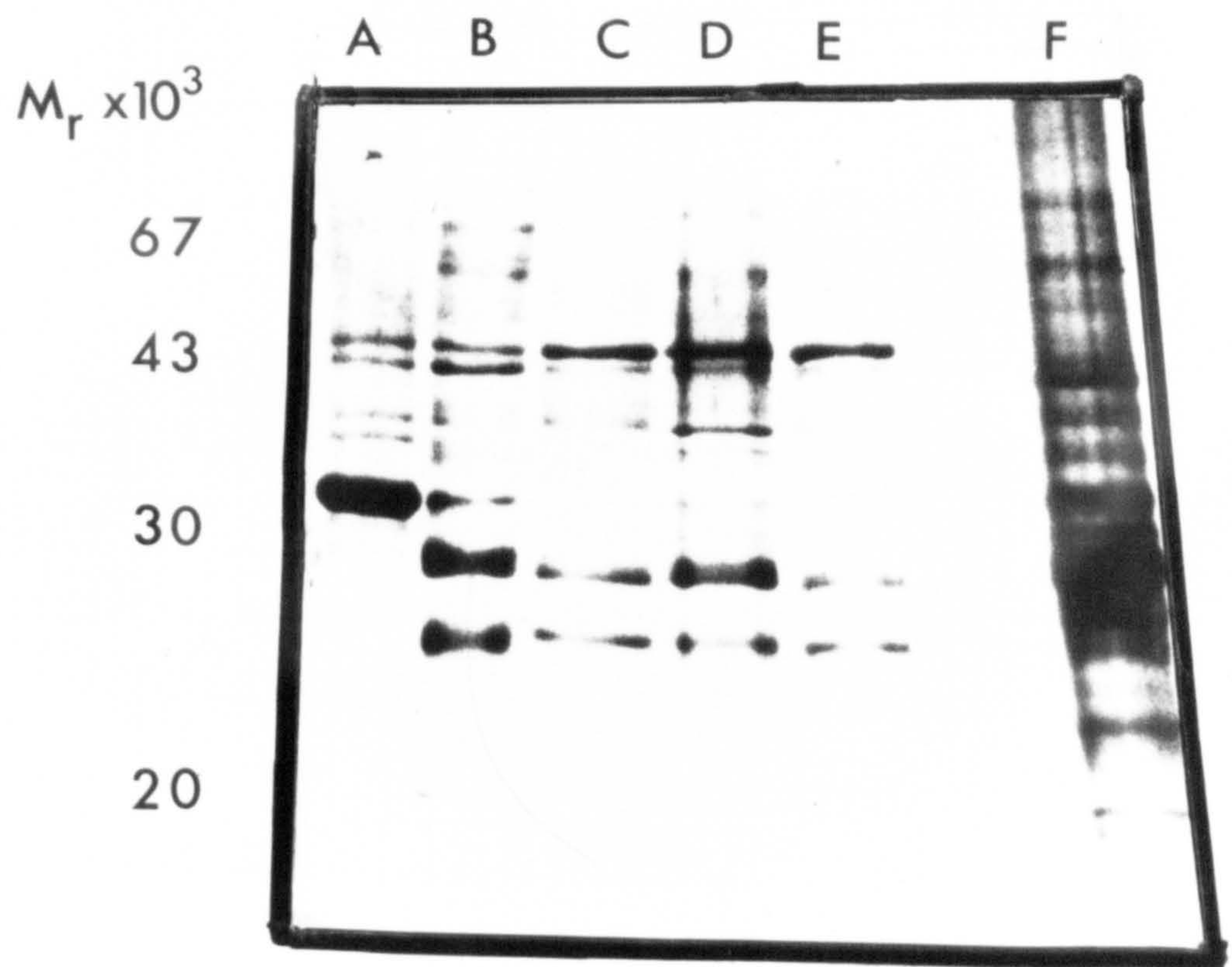
However, as both Miller et al. (1984a) and Turpin et al. (1985b) found, most experiments on the kinetics of Ci uptake showed that the kinetics did not follow simple Michaelis-Menton type affinities, and the results shown here are from graphs that most closely fitted Michaelis-Menton kinetics. Turpin et al. (1985b) found in those cultures where the Ci-concentrating mechanism was repressed, a simple Monod relationship could not be used to describe growth in relation to DIC, and as has already been shown, in none of the chemostat cultures used in this study was the Ci-concentrating mechanism fully induced.

Figure 4.10 shows the polypeptide profile from Mr 67,000 to Mr 20,000 after SDS-PAGE, of the cytoplasmic membrane fractions isolated from chemostat cultures grown over a range of DIC concentrations. Track F (from a chemostat culture gassed with 5% CO<sub>2</sub>) has deliberately been overloaded to emphasize more strikingly the most important aspect of the results, namely that in all the chemostat cultures gassed with air, where DIC was limiting for growth, the 42 kD polypeptide is present, whilst in



Figure 4.10 : SDS-PAGE of cytoplasmic membrane fractions from chemostat cultures of *Synechococcus* PCC7942

10-30% exponential gradient gel run at 20mA, 4°C for 16 hours. Track A, Low CO<sub>2</sub> batch culture; Track B, Air continuous culture; Track C, 40% Air continuous culture; Track D, 20% Air continuous culture; Track E, 17% Air continuous culture; Track F, 5% CO<sub>2</sub> continuous culture.





the cultures in which DIC was in excess it is not present. As has already been demonstrated, the DIC concentration in the chemostats used in this study never reached a point where it was limiting for the Ci concentrating mechanism, and yet the 42 kD polypeptide, at one time linked with the Ci concentrating mechanism (Omata and Ogawa, 1986), is still present. This reinforces in physiological terms, what has already been shown in molecular terms by Schwartz *et al.* (1988) and Omata *et al.* (1990), namely that there is no direct causal link between the 42 kD polypeptide and the Ci concentrating mechanism. Reddy *et al.*, (1989) have cloned and sequenced the gene (*cbpA*) encoding a 42 kD cytoplasmic membrane carotenoprotein in *Synechococcus* PCC7942, and sequence analysis has shown this to be the same 42 kD polypeptide as that cloned and sequenced by Omata *et al.*, (1990). Using Northern blot analysis, Reddy *et al.*, (1990) have shown that transcription of *cbpA* is tightly regulated by light intensity and iron concentration, with transcription being greatly induced by growth at high light intensities, leading the authors to conclude that at least one function for this carotenoid-protein association may be the protection of cells against photooxidative damage, although the mutant strain constructed by Omata *et al.*, (1990) by inserting a kanamycin cartridge into *cbpA* grew normally under high light intensities as well as low CO<sub>2</sub> conditions. If it does indeed prove to be the case that the 42 kD polypeptide is induced under high light and low CO<sub>2</sub> growth conditions, it could help explain the results shown in Fig. 4.10, because as CO<sub>2</sub> decreased in the chemostat,

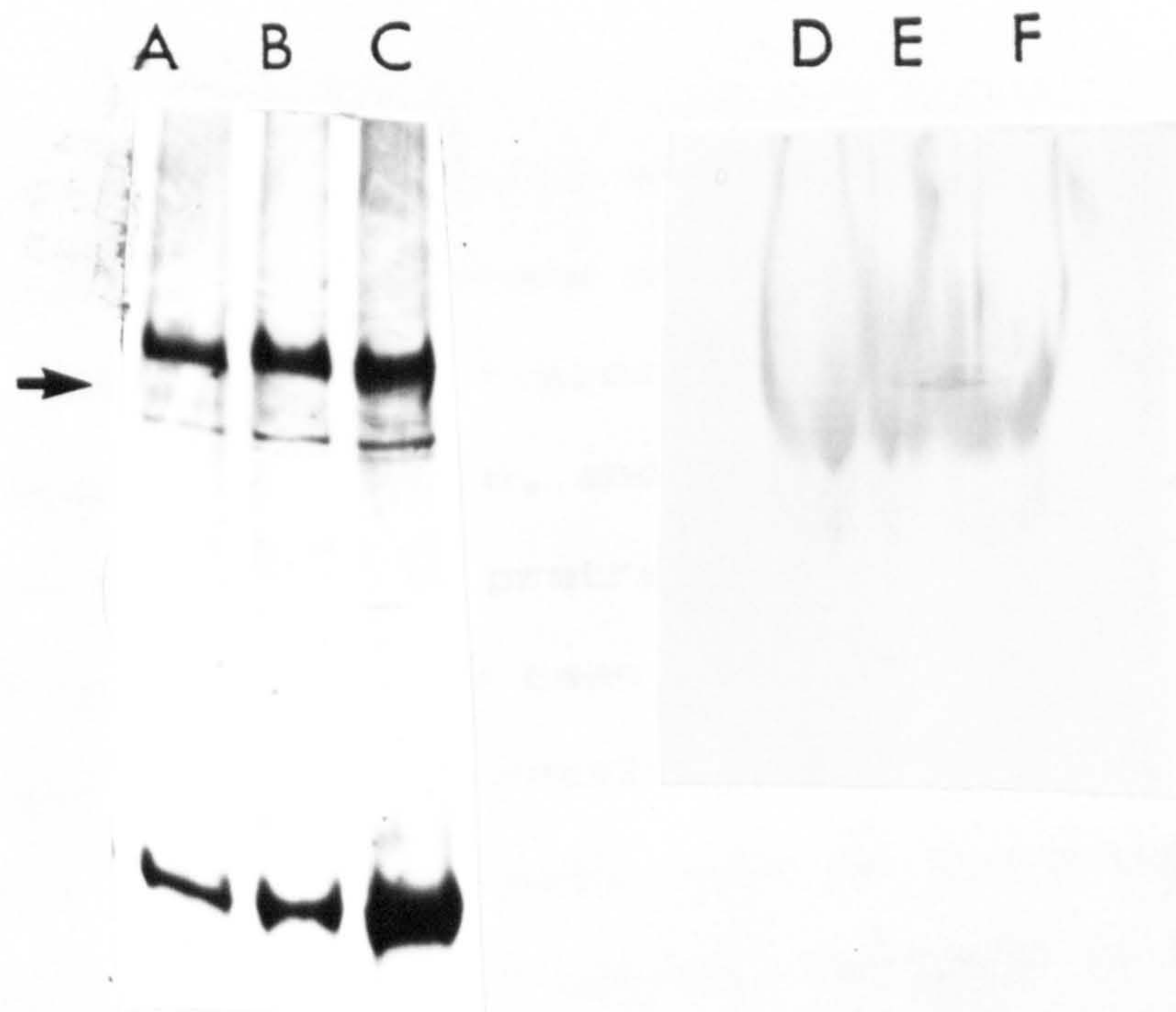
causing a drop in biomass, the incident light intensity would obviously increase, and the 42 kD polypeptide increases as biomass decreases (see Fig 4.10). However, as suggested by Reddy *et al.*, (1990) it may also be the case that transcription from *cbpA* is induced under a variety of environmental stresses, and thus be considered a generalized stress protein.

In an attempt to try and establish whether the increase in RuBisCO activity seen in chemostat cultures as DIC became limiting for growth was due to increased activity *per se*, as suggested by the discontinuous alteration seen in RuBisCO activity, or rather due to increased amounts of the enzyme, cell-free extracts from chemostat cultures grown under different DIC regimes were subjected to non-denaturing PAGE (see Fig. 4.11). RuBisCO was identified by running parallel samples on the same gel, the position of the enzyme identified by Western blotting using anti-RuBisCO antibody (a kind gift from S. van der Vies, this laboratory). By comparing the distance migrated on the Western blot to that on the silver stained gel, the position of RuBisCO was identified on the silver stained gel (arrowed). As the anti-RuBisCO antibody did not have  $^{125}\text{I}$  bound to it, it does not allow RuBisCO to be quantified accurately, however the detection system used does allow a visual indication of the amount of RuBisCO present, and because protein loading was the same in each track and the concentration of the anti-RuBisCO antibody used was also the same, it does allow for crude comparisons. As can be seen from Fig. 4.11, it would



Figure 4.11 : Western blot of cell-free extracts from chemostat cultures of *Synechococcus* PCC7942 showing RuBisCO

Non-denaturing 7.5-15% PAGE of cell-free extracts from chemostat cultures gassed with 5% CO<sub>2</sub> (tracks A+D), 0.03% CO<sub>2</sub> (tracks B+E) and 0.0051% CO<sub>2</sub> (tracks C+F). Tracks A,B & C have been stained with silver ions, tracks D,E & F represent the Western blot of parallel samples using RuBisCO antibody (1:1000 dilution) and a peroxidase conjugated goat anti-rabbit 2<sup>o</sup> antibody detection system.





appear that there is little difference in the amounts of RuBisCO under the various growth conditions in both the silver stained gel and Western blot, suggesting that the changes seen in RuBisCO activity represent increased activation of the enzyme rather than increasing quantities of the enzyme, although obviously this will have to be qualified using a more quantifiable method.

#### 4.4 Conclusion

This work has shown the difficulty involved in obtaining large quantities of cytoplasmic membrane using the floatation centrifugation method, yields typically averaging between 0.02 - 0.04% of the total cell protein, and the ineffectiveness, with the exception of lysozyme, of pretreatments at increasing the yield. Although it has already been mentioned that the 42 kD cytoplasmic membrane is not directly linked to the  $\text{Ci}$ -concentrating process, obviously some of the proteins which are involved in the process may well be resident in this membrane fraction. It has already been shown in this laboratory that to obtain the 42 kD polypeptide, one of the more abundant polypeptides in the cytoplasmic membrane fraction, in sufficiently large quantities to begin purification, that 20 litre fermentors have to be used (J. Mason, per. comm.), suggesting that fermentors on this scale at least, will have to be used for any other polypeptides isolated in this manner. Work in chemostats gassed with varying combinations of  $\text{CO}_2$ :air: $\text{CO}_2$ -free air has shown that the term DIC-limited must be

used carefully, because it has been shown that although cultures have been DIC-limited for growth (using classical chemostat definitions), they have not actually been DIC-limited with respect to the  $C_i$ -concentrating mechanism. RuBisCO activity increased as the external DIC concentration fell, however this increase did not occur stepwise, but in three distinct phases. It would be interesting to see the RuBisCO activity in chemostat cultures in which the  $C_i$ -concentrating mechanism was fully induced, and obviously this is work which can be performed in the future. A preliminary investigation into whether or not this increase in RuBisCO activity was due to increased amounts of the enzyme, or an actual enzyme activation suggested that there was an actual enzyme activation, although more detailed work will have to be performed to verify this. The anti-RuBisCO antibody used in this study to identify RuBisCO could be used in this process, as the antibody-protein reaction is quantifiable if it is linked to  $^{125}I$ , however, unfortunately for this study, there was insufficient antibody to allow this.

The lack of correlation between the presence of 42 kD cytoplasmic membrane polypeptide and the induction of the  $C_i$ -concentrating process has been shown at a physiological level in this study. Although molecular microbiological methods are now at the forefront in proving/disproving causal relationships between physiological events and the proteins (genes) controlling them, the use of purely physiological methods, such as chemostat culture should not be ruled out, especially as it allows growth under defined substrate conditions, which the majority of

experiments, carried out in batch culture, do not.



## Chapter 5

*In vivo* and *in vitro* protein phosphorylation in  
*Synechocystis* PCC6803 : Responses to changes in  
the growth regime/assay conditions

## 5.1 Introduction

### 5.1.1 Post-translational modification

Translation, in biological terms, is the process of assembling amino acids into proteins, the exact sequence being specified by the nucleotide sequence of messenger RNA molecules. The term post-translational modification is used to describe any alteration to an amino acid or polypeptide after its polymerization.

As such, post-translational modification encompasses three types of events. (Uy and Wold, 1977). The first involves weak, non-covalent interactions resulting in the folding of the polypeptide chain and the association of chains with each other, and with non-covalently bound ligands. The second involves the transport of the synthesized polypeptide to its site of action, and the third involves various covalent modifications, which can be functional or regulatory. In the cell these three physically and chemically distinct protein processing steps are integrated into a single continuous process.

The role of post-translational covalent modification of proteins as a means of metabolic regulation is well documented. There are two distinct types of covalent modification, peptide bond cleavage and amino acid derivitization.

The former, as it is described involves the cleavage of peptide bonds to convert long chain, generally inactive precursors, to shorter chain, active proteins. All bacterial proteins are synthesized with N-formylmethionine as the first amino acid, however few retain this N-terminal formyl group. This cleavage

is fairly non specific, since overproduction of a new protein often results in a mixture of formylated and deformylated forms. A more specific cleavage occurs when proteins are translocated through or inserted into membranes, which results in the removal of an amino-terminal or leader sequence (see Wickner and Lodish, 1985). In eukaryotes, many enzymes are known to be activated by proteolytic cleavage; peptide bond cleavage was originally discovered in the proteolytic enzymes, pepsin, trypsin and chymotrypsin (see Kraut, 1977). In these enzymes the inactive precursor (or zymogen) is activated by cleavage of a single peptide bond, although in blood coagulation, blood clots are formed by a series of zymogen activations (see Jackson and Nemerson, 1980).

Covalent protein modifications can be split into three categories (Wold, 1981): modifications involving either carboxy-terminal, amino-terminal or individual amino acid side chains. The extent of this post-translational covalent modification of proteins is substantial. Uy and Wold (1977) found 140 amino acids and derivatives as constituents of various proteins in various organisms, and by 1981 this had risen to several hundred (Wold, 1981), yet only 20 amino acids are specified in the genetic code.

Modifications of the carboxy-terminal group are quite uncommon, restricted to only a few proteins including histone H1, which is known to be ADP-ribosylated on the carboxyl-terminal lysine residue (see Wold, 1981). There are a larger number of modifications made on the amino-terminal residue of a protein,



by far the most common being N-terminal acylation (see Driessen et al., 1985). A large number of animal and plant proteins are N-terminally acylated, along with a few bacterial proteins, of which the majority are ribosomal proteins. Modifications of the amino acid side chains are the largest category of covalent modifications. There are many forms of covalent modification, including methylation, halogenation, ADP-ribosylation and many other specific amino acid modifications, most of which are uncommon (see Uy and Wold, 1977; Wold, 1981). However the largest categories of covalent modifications are glycosylation and phosphorylation. Glycosylation, the introduction of covalently linked monosaccharides, oligosaccharides and polysaccharides, is the most common modification, however its exact function is unclear. It is thought that one of the roles of the sugar moieties is in biological communication involving cell-cell and cell-molecule interactions, such as the requirement of specific oligosaccharide sequences by lectins for tight binding to occur. Amongst prokaryotes, glycosylation also occurs, the carboxysomes of *Thiobacillus neopolitanus* have been found to contain glycoproteins (Holthuisen et al., 1986), and four phycobilisome associated glycoproteins were found in the cyanobacterium *Anacystis nidulans* (Reithman et al., 1987). Of all the types of covalent modification, protein phosphorylation/dephosphorylation is the best studied example, is the subject of this chapter, and is discussed below.

### 5.1.2 Protein phosphorylation

The direct involvement of protein phosphorylation in a metabolic pathway was first established in 1956 by Krebs and Fischer, who found the activity of rabbit skeletal muscle glycogen phosphorylase was regulated by reversible phosphorylation.

In recent years, many proteins have been found to be the subject of phosphorylation/dephosphorylation. In eukaryotic systems, the process has been shown to play a key role in several cellular functions including metabolic pathways, synthesis and organization of cell constituents, gene expression and control of cellular proliferation (see Rubin and Rosen, 1975; Krebs and Beavo, 1979). Not only is protein phosphorylation involved in the control of many specific cellular functions, it has also become clear that it is also the basis of a complex network of interlocking systems which control and coordinate multiple diverse biological functions, acting through a small number of second messengers, including cAMP, cGMP and calcium (Cohen, 1986). Other regulatory agents, producing a change in the phosphorylation state of a specific protein include steroid hormones, insulin, interferon, thyroid hormone, hemin, viruses and light (Greengard, 1978).

Amongst eukaryotes, a single type of protein phosphorylating system, the ATP-dependent protein kinase, is currently believed to be the sole mediator of phosphorylation-mediated regulatory responses (see Figure 5.1). Although ATP is generally the phosphoryl donor, GTP has also been documented (Nimmo and Cohen, 1974, as cited by Krebs and Beavo, 1979).

### 5.1.3 Prokaryotic protein phosphorylation

The earliest evidence of protein phosphorylation in prokaryotes came from Kuo and Greengard (1969). They reported on the presence of an enzyme in *E.coli* cell extracts that could catalyze the phosphorylation of exogenous histones and basic proteins. This report was followed by others (Gordon, 1971; Kurek et al., 1972; Khandelwal et al., 1973), however all of these reports failed to provide evidence on the nature of the phosphorylated moiety of the proteins, and exogenous proteins were used as substrates instead of endogenous ones.

The existence of kinases in prokaryotes was indicated by other studies: a polyphosphate kinase was described in *E.coli* (Li and Brown, 1973) and an acylphosphate kinase in *Caulobacter crescentus* (Agabian et al., 1972).

The first clear demonstration of a protein kinase activity in prokaryotes was observed from analysis of bacteriophage T7 infection in *E.coli* (Ramsdorf et al., 1974). An enzyme induced upon infection could phosphorylate endogenous and exogenous proteins, and the products of the reaction had the chemical characteristics of phosphoserine and phosphothreonine, however the authors simultaneously showed the kinase to be coded for by the viral genome. Hence Rubin and Rosen (1975) concluded there was no protein kinase activity in prokaryotes. This idea was abandoned in 1978, when phosphorylation of proteins by protein kinases was reported in *Salmonella typhimurium* by Wang and Koshland and in *Escherichia coli* by Manai and Cozzone (1979).



The first identification of an endogenous substrate for a protein kinase was made by Garnak and Reeves (1979a). They observed that the addition of acetate to a stationary phase culture of *E.coli* grown in the presence of  $^{32}\text{P}$ -orthophosphate resulted in the incorporation of radiolabelled phosphate into the enzyme isocitrate dehydrogenase, concomitant with a loss of isocitrate dehydrogenase activity. Enami and Ishihama (1984) found more than 40 polypeptides species were phosphorylated in *E.coli* *in vitro* as well as *in vivo* at serine and threonine residues. They purified two types of protein kinase, one which phosphorylated an endogenous protein and another capable of autophosphorylation.

Although the majority of work on prokaryotic protein phosphorylation has been carried out in *E.coli*, protein phosphorylation has been demonstrated in a number of bacterial species. In most cases phosphoproteins have been detected by their ability to incorporate radiolabelled phosphate *in vivo* from  $^{32}\text{P}$ -orthophosphate or *in vitro* from gamma labelled [ $^{32}\text{P}$ ]ATP. These include *Halobacterium halobium* (Spudich and Stoekkenius, 1980), *Myxococcus xanthus* (Komano et al., 1982), *Streptococcus pyogenes* (Deutscher and Saier, 1983), *Rhodospirillum rubrum* (Holmes and Allen 1986, Holmes and Allen, 1988), *Rhodospirillum rubrum* (Turner and Mann, 1986) and *Streptomyces coelicolor* (Stowe et al., 1989). Protein phosphorylation has been reported in three species of cyanobacteria. Most work has concentrated on phosphorylation of the photosynthetic apparatus in *Synechococcus* 6301 (see Allen et

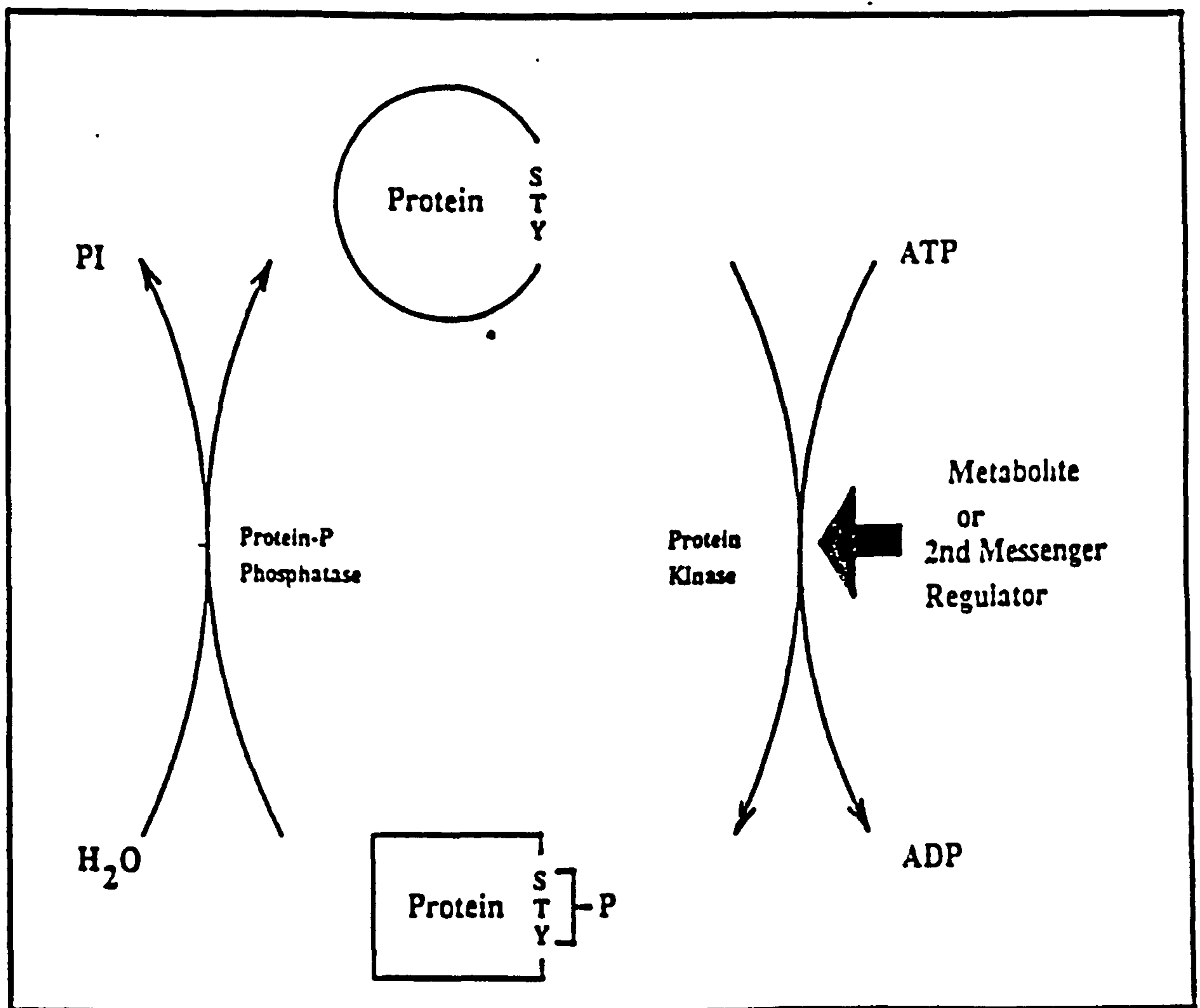
al., 1985; Sanders et al., 1986), the two other species being *Freneyella diplosiphon* (Schuster et al., 1984) and *Anabaena* PCC7120 (Mann et al., 1991).

As already mentioned, a single type of protein phosphorylating system is employed by eukaryotes, the ATP-dependent protein kinase (see Fig. 5.1). Prokaryotes also possess protein kinases, the roles of which superficially resemble eukaryotic protein kinases, phosphorylating seryl, threonyl or tyrosyl residues in target proteins, preferentially using ATP as the phosphoryl donor. These protein kinases regulate intermediary metabolism, carbohydrate transport, gene transcription and cellular differentiation, and they also appear to regulate bacterial specific processes such as bacteriophage infection, virulence and bacterial photosynthetic carbon dioxide fixation (for a full review see Cozzzone, 1988).

Most of these bacterial protein kinases are allosterically activated by cellular metabolites rather than second messengers (Reizer et al., 1988), although regulation by  $\text{Ca}^{2+}$  has also been reported (Turner and Mann, 1989). The best characterized of the metabolite regulated bacterial protein kinases are the isocitrate dehydrogenase kinases of enteric bacteria, and the kinases which phosphorylate HPr, a phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) of Gram positive bacteria, both of which are dealt with in detail in Section 5.1.4.

In addition, two other protein phosphorylating systems operate

Figure 5.1 : Schematic illustration of the classical metabolite, second messenger, activated protein kinases. These enzymes phosphorylate serine (S), threonine (T) or tyrosine (Y) residues in target proteins.  
 (From Saier et al., 1990).





in prokaryotes apart from the classical protein kinases, having no counterparts, as of yet, in the eukaryotic kingdom. One of these is the sensor kinase/response regulator system, in which, in response to a stimulus, a histidyl residue within a conserved C-terminal domain of a sensor kinase is autophosphorylated, and the phosphoryl group then transferred to an aspartyl residue within a conserved N-terminal region of a second protein. These sensor kinase/response regulator systems vary in complexity from a single polypeptide, such as that which controls motility and development in *Myxobacteria*, through to systems which consist of several protein components which allow response to a wide variety of stimuli, and regulate such divergent prokaryotic processes as chemotaxis, nitrogen fixation, sporulation, virulence and osmosensitivity, amongst others. For a full review of this system see Stock et al. (1989).

The third type of prokaryotic kinase system is the well characterized and extremely complex PTS system. In this system a phosphoryl group donated by phosphoenolpyruvate is passed down a chain of cytoplasmic and membrane bound phosphorylatable proteins, mediating group translocation of imported carbohydrates. The PTS system also operates as a chemoreceptor system in chemotaxis towards carbohydrates, and as a pacemaker regulatory system for transcription of operons necessary for the catabolism of non-PTS sugars. For a more detailed review of the PTS see Section 5.1.4.3, and Postma and Lengeller (1985); Saier (1989); Meadow et al. (1990).

#### 5.1.4 Biological role of phosphorylation

Phosphorylation/dephosphorylation within prokaryotic cells, was discovered by analogy to the situation in eukaryotes, an indication that reversible phosphorylation could represent a regulatory device for controlling cellular functions in prokaryotes.

##### 5.1.4.1 Isocitrate dehydrogenase

The best studied example of the cellular processes occurring within prokaryotes under the regulation of protein kinases and phosphoprotein phosphatases is the control of *E.coli* isocitrate dehydrogenase (ICDH) activity. During growth on acetate as the sole carbon source, the bacteria induce the glyoxylate bypass enzymes, isocitrate lyase and malate synthase, which divert some of the carbon flux through the glyoxylate cycle, providing energy and intermediates for the biosynthetic pathway. If this did not occur, both of the carbon atoms from acetate would enter the Krebs cycle, be converted to carbon dioxide, leading to an exhaustion of metabolites. Thus when acetate must serve as the sole carbon and energy source, channeling of metabolites from the Krebs cycle into the glyoxylate bypass occurs at isocitrate (see Kornberg, 1966) (Fig. 5.2).

The first evidence implicating phosphorylation in the control mechanism came from Garnack and Reeves (1979a), who observed that concomitant with a loss of ICDH activity during growth on acetate was the incorporation of  $^{32}\text{P}$ -phosphate into the enzyme.

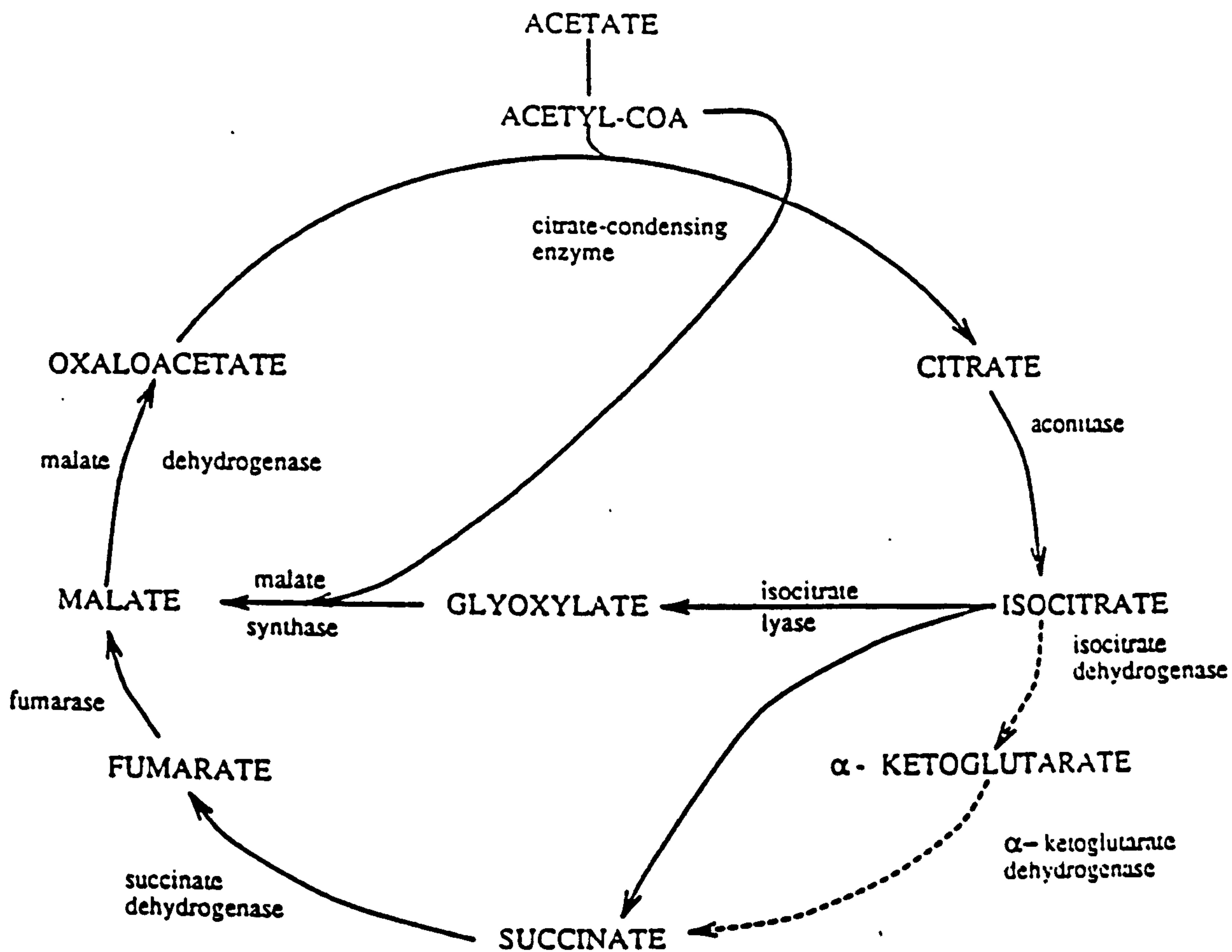


Figure 5.2. The TCA cycle and glyoxylate bypass. Dashed lines indicate reactions not occurring in the glyoxylate bypass.



Conversely, when grown on glucose, or other carbon sources that do not use the glyoxylate bypass, ICDH is fully active and dephosphorylated. Immunological techniques confirmed that the phosphorylated and non-phosphorylated forms of the enzyme were identical, and partial acid hydrolysis allowed the identification of phosphoserine (Garnack and Reeves, 1979b). LaPorte and Koshland (1982) found that phosphorylation and dephosphorylation are catalysed by a single bifunctional enzyme, ICDH kinase/phosphatase.

Considerable information has been obtained concerning the regulation of ICDH in *E.coli* (see Nimmo, 1984). It is the only phosphorylation system in prokaryotes in which the understanding of the molecular details is comparable to eukaryotic systems. Nimmo and Nimmo (1984), found on investigation of the regulatory properties of ICDH kinase/phosphatase that the kinase was inhibited by several metabolites which stimulated the phosphatase, indicating that the system is very sensitive to variations in the intracellular concentration of metabolites. It was suggested by Nimmo and Nimmo (1984) that phosphorylation of ICDH, as a result of growth on acetate, rendered it rate limiting to the TCA cycle, and as a result isocitrate accumulates and is diverted through the glyoxylate bypass.

#### 5.1.4.2 Citrate lyase ligase

In *Clostridium sphenoides*, citrate can be utilized anaerobically to produce L-glutamate, or degraded through a fermentative pathway when L-glutamate is available (Antranikian et al.,

1984). The breakdown of citrate is catalyzed by citrate lyase, an enzyme which can exist in two forms, a sulfhydryl (inactive) and an acetyl (active) form. The conversion from an inactive to an active form of citrate lyase is catalyzed by citrate lyase ligase, the activity of which is reported to be regulated by phosphorylation/dephosphorylation (Antranikian et al., 1985). In a medium deplete of citrate, citrate lyase ligase is inactivated by dephosphorylation, consequently citrate lyase can no longer be activated. Upon addition of citrate or L-glutamate, citrate lyase ligase is activated by phosphorylation, resulting in the activation of citrate lyase.

#### 5.1.4.3 Regulation of metabolite transport

The bacterial phosphoenolpyruvate-dependent phosphotransferase system, mentioned earlier in this chapter, catalyzes the concomitant phosphorylation and translocation (group translocation) of certain carbohydrates across the bacterial cytoplasmic membrane. It consists of four proteins, enzyme I, HPr, factor III and enzyme II (see Postma and Lengeler, 1985). The transport of carbohydrates requires the following sequence of reactions; i) transfer of the phosphate group from phosphoenolpyruvate (PEP) to a histidyl residue of enzyme I; ii) transfer of this phosphate group to a histine residue in HPr; iii) The same phosphoryl group is transferred from HPr to a histidyl residue in factor III, and finally iv) the phosphoryl group is transferred from factor III to the sugar about to be transported into the cell, catalyzed by enzyme II, which is

membrane associated. Factor III and enzyme II are specific for a certain carbohydrate (see Postma and Lengeler, 1985).

HPr can be phosphorylated at a single serine residue in a number of *Streptococcus* species, the reaction is catalyzed by a membrane-associated protein kinase. The kinase from *Streptococcus pyogenes* has been shown to phosphorylate HPr's of various Gram-positive bacteria, but not *E.coli* (Reizer et al., 1984). The phosphorylated HPr is unable to transfer its histidyl phosphate group to a carbohydrate as a part of the PEP-phosphotransferase system. When the HPr is phosphorylated at its serine residue by ATP-dependent phosphorylation, its PEP-dependent phosphorylation by enzyme I at a histidine residue is slowed down by a factor of 5000 in *in vitro* systems (Deutscher et al., 1984). This ATP-dependent phosphorylation of HPr at the serine residue is a regulatory event to control carbohydrate uptake, whereas the PEP-dependent phosphorylation by enzyme I is part of the bacterial phosphotransferase system (Deutscher et al., 1984).

#### 5.1.4.4 Regulation of photosynthesis

Photosynthetic organisms can, in response to changes in the spectral quality of incident light perform short-term adaptations known as State I-State II transitions (Bennett, 1977; Allen, 1983). Transition to State II occurs when conditions favour preferential excitation of photosystem II (PSII), and results in the transfer of excitation energy away from PSII in favour of PSI. Transition to state I occurs under



the reversal of this situation, the net result being that the quantum efficiency of photosynthesis is maintained despite variations in the light regime (for review see Williams and Allen, 1987).

In green plants light harvesting is accomplished by intrinsic membrane protein complexes (LHC-I and II), binding Chl-a and Chl-b. The phosphorylation state of the light-harvesting pigment-protein complex LHC-II regulates the distribution of excitation energy between photosystems I and II (PS-I and PSII) (Allen et al., 1981). Phosphorylation of LHC-II is thought to lead to its dissociation from PS-II and its association with PS-I (Kyle et al., 1984).

Cyanobacteria are devoid of the light-harvesting chlorophyll a/b binding proteins (LHC's), but are still capable of regulating excitation energy by state I-state II transitions (Olive et al., 1986). Allen et al. (1985), working with *Synechococcus* 6301, reported on a light dependent phosphorylation of two polypeptides of 18,500 and 15,000 kD, the former found in the soluble fraction and the latter associated with the membranes, and it was suggested that distribution of absorbed energy might be regulated by phosphorylation of membrane proteins. Other studies using the same organism demonstrated that the two polypeptides were phosphorylated only upon illumination (Sanders et al., 1986). In 1988, again in *Synechococcus* 6301, Sanders and Allen reported that light-dependent phosphorylation of a 15 kD polypeptide from the thylakoid membrane was dependent on divalent cations and that this protein did not become

dephosphorylated in the dark. Although their results from fluorescence emission spectroscopy are contrary to their previous work (Allen et al., 1985, see above) indicating that changes in fluorescence emission do not arise from a transition to State II, they still consider membrane protein phosphorylation may play a role in state transitions in cyanobacteria. More recently, light-dependent protein phosphorylation has been observed in isolated thylakoid membranes of *Synechococcus* PCC6301 (Harrison et al., 1991). In particular a polypeptide of 15 kD was shown to be preferentially phosphorylated under plastoquinone-reducing conditions, a situation analogous with the higher plant chloroplast (see Allen et al., 1981)

*Rhodospirillum rubrum* has also been shown to exhibit protein phosphorylation. Holuigue et al. (1985), reported on a number of proteins that were phosphorylated *in vitro* in a cell free system, including two of 13 kDa and 11 kDa (by SDS-PAGE) appropriate for the chlorophyll-antenna apoproteins of B880. Holmes and Allen (1986), using  $^{32}\text{P}$  labelling *in vivo*, investigated these observations further and found two polypeptides of 17,000 kDa and 13,000 kDa that were phosphorylated under cooperative conditions, and one polypeptide of 10,500 kDa was phosphorylated under non-cooperative conditions. The polypeptides of 13,000 kDa and 10,500 kDa were identified as B880- $\alpha$  and B880- $\beta$  respectively. Allen and Holmes (1986) proposed a general model for the regulation of photosynthetic units function by protein phosphorylation, which

can be applied to PSII of green plants, algae and cyanobacteria, as well as to the single photosystem of purple non-sulphur bacteria. Regulatory effects of membrane protein phosphorylation in photosynthetic systems result from simultaneous phosphorylation by a single kinase of the polypeptides of two intrinsic pigment-protein complexes. One complex is a peripheral light-harvesting complex and the other is bound to the reaction centre and functions as a link in excitation energy transfer. When phosphorylated these polypeptides are electrostatically repelled in a direction parallel to the membrane plane and their excitation energy is decreased between them, thus phosphorylation decreases the cooperativity of photosynthetic units.

Mann and Turner (1988) have reported that in *R. vannielli* the large subunit of RuBisCO, the key enzyme of CO<sub>2</sub> fixation, is phosphorylated and that this phosphorylation occurs prior to assembly of the large subunit into the mature enzyme. They further suggest that this phosphorylation may play a role with the assembly of the mature enzyme.

Using synchronously grown *C. reinhardtii*, (Marcus et al. 1986) showed that the activity of the CO<sub>2</sub> concentrating mechanism, including the ability to concentrate CO<sub>2</sub> internally and the activity of carbonic anhydrase peaked after six to nine hours of light and reached a minimum after the same period of dark. There was also a similar increase in the capacity for *in vitro* phosphorylation of thylakoid proteins, which reached a maximum after nine hours of light and decreased towards the dark period.



The authors believed that there was an interaction between the internal (cell cycle) and the external (light) signals, the rythmical alterations in photosynthetic  $V_{max}$  resulting from possible changes in PSII activity.

#### 5.1.4.5 Regulation of the cell cycle

Protein phosphorylation has been shown to be involved in various stages of the cell cycle. Cortay et al. (1986) suggested that protein phosphorylation becomes more active, or dephosphorylation is inhibited as cell division slows down. Turner and Mann (1986) found, on comparison of the phosphopolypeptide pattern of reproductive and swarmer cell populations of *Rhodospirillum rubrum*, that phosphorylation was almost absent in swarmer cells, whilst a number of phosphopolypeptides were present in reproductive cells, and they suggested that protein phosphorylation may play a role in differentiation.

The developmental cycle of *M.xanthus* is characterized by the formation of mounds of mature spores, referred to as fruiting bodies, in a nutrient-depleted medium. Spore formation can also be induced chemically by the addition of glycerol (Kaiser et al., 1979). Upon chemical induction (using glycerol) of myxospore formation, Komano et al. (1982), found that the pattern of protein phosphorylation changed. Two membrane polypeptides were newly phosphorylated 1-2 hours after induction of sporulation, which suggested that protein phosphorylation may be required for sporulation, and this has recently been proven

(see Burbulys et al., 1991).

Protein phosphorylation in prokaryotes, whilst by no means as well studied as in eukaryotes, is a rapidly expanding field of study, being observed in a wide range of prokaryotic species, under a wide variety of growth conditions, suggesting that phosphorylation may prove a universal form of regulation amongst all life forms.

#### 5.1.5 The nature of phosphorylated amino acid residues

Phosphorylated amino acid residues in proteins are commonly divided into three groups (Martenson, 1984): i) *O*-phosphomonoesters - formed by phosphorylation of the hydroxyamino acids, serine, threonine and tyrosine. They all resist acid and hydroxylamine treatment, but with the exception of phosphotyrosine, are cleaved by alkali; ii) *N*-phosphates - produced by phosphorylation of the basic amino acids arginine, histidine and lysine. They are hydrolysed by hydroxylamine and acid; iii) Acyl phosphates - generated by phosphorylation of the acidic amino acids aspartic acid and glutamic acid. They are extremely acid and base labile, and are destroyed by hydroxylamine. In the work described above, most authors have made no attempt to distinguish the different type of phosphate linkage, and as in eukaryotes, the most commonly employed assay procedures and isolation methods of phosphoproteins in prokaryotes involve the use of acid (see Martenson, 1984). This destroys *N*-phosphates and acyl

phosphates, leaving only ester-linked phosphates. Although there is no evidence to suggest that only ester-linked phosphorylation has any regulatory significance, it does so far seem to be the general case (Cozzzone, 1984).

We have confined our studies to looking for ester-linked phosphopolypeptides, since the inclusion of acyl-linked phosphopolypeptides would hinder visualization of regulatory phenomena, due to the role of acyl-phosphoprotein linkages as intermediates in certain enzymatic mechanisms such as the two-component signal transduction system. There is the possibility that nucleotidylation can lead to errors in the characterization of phosphoamino acids. This occurs when phosphoryl groups act as bridging moieties in the phosphodiester linkages of nucleotide to protein (Martenson, 1984). This type of modification has been found in adenylylated glutamine synthetase (Caban and Ginsberg, 1976) and in uridylylated P11 regulatory protein in *E.coli* (Rhee et al., 1985). It is also possible that some phosphate labelled bands on gel autoradiographs are the result of ADP-ribosylation, although it appears to occur less extensively in prokaryotes than phosphorylation. Indeed, Gaal and Pearson (1986) in their review of ADP-ribosylation state "There is little evidence to suggest that ADP-ribosyl transferases exist in uninfected bacterial cells". However, it has been shown by Pope et al. (1985) that the nitrogenase iron protein of *R.rubrum* is ADP-ribosylated *in vivo*, and can be reversibly regulated by ADP-ribosylation *in vitro* (Lowey et al., 1986). ADP-ribosylation is sensitive to



treatment with neutral hydroxylamine, which degrades (ADP-ribosyl)-protein linkages. However, 10-30% of linkages are resistant to this treatment, and are only labile in hot alkali (Burzio, 1982), which also destroys most phosphomonoester linkages. Thus there are no easy methods to distinguish between phosphorylation and ADP-ribosylation, enzymatic methods that may place constraints upon experimental procedures being the only possibility.

The concentration of Ci within the cell requires a large expenditure of energy (see Raven and Lucas, 1985), and consequently it is probably a tightly regulated process. In chapter one the interlink between certain metabolic pathways was discussed, and in chapter three it was shown that nutritional factors apart from the CO<sub>2</sub> regime could affect expression of the Ci concentrating mechanism. In view of the fact that protein phosphorylation is important in the regulation of many metabolic processes (see Section 5.1.4), the aim of the work in this chapter was to study protein phosphorylation in selected unicellular cyanobacteria in response to changes in the CO<sub>2</sub>-regime, to see whether the loss of the Ci concentrating mechanism correlated temporally with changes in the pattern of protein phosphorylation.

## 5.2 Materials and methods

The specific procedures for *in vivo* and *in vitro* labelling and phosphoprotein gel treatment are detailed in section 2.16

### 5.2.1 Isolation of phycobilisomes

Phycobilisomes were isolated from *Synechococcus* PCC6803 using the method of Yamanaka et al. (1978). All buffers contained 1 mM 2-mercaptoethanol and 1 mM sodium azide, and unless otherwise stated all procedures were carried out at room temperature. Mid-exponential phase cells (100 ml) were harvested by centrifugation, washed twice in 0.65 M  $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (Na-K-P) buffer (pH 8.0), resuspended in 5 ml of the same buffer, and disrupted by two passages through a French pressure cell at 24,000 p.s.i. The cell lysate was then incubated in the presence of 1% (v/v) Triton X-100. Whole cells and membrane particles were removed by centrifugation at 8000 rpm for 30 min at 18°C, in an HS-21 centrifuge (Beckman). The intensely blue supernatant formed as a middle layer between the pelleted cells/membrane fractions and the green membrane/detergent layer, was carefully removed, 0.2 ml stored for absorption spectra and SDS-PAGE analysis, and the rest layered in 1 ml aliquots onto sucrose step gradients in 14 ml polyallomer tubes consisting of; 2.0, 3.0, 3.0, 2.5, 2.5 ml, of 2.0, 1.0, 0.75, 0.5, and 0.25 M solutions of sucrose, all in 0.75 M Na-K-P, pH 8.0. These were then placed in a Beckman SW40 rotor, and centrifuged for 16h at 24,000 rpm, 18°C. The phycobilisomes were recovered as a deep blue band from the 0.75 M sucrose layer (Abbe refractometer),

fractions analyzed by absorption spectrometry and SDS-PAGE, and the remainder frozen at  $-20^{\circ}\text{C}$  until required.

#### 5.2.2 Partial purification of phosphorylated polypeptides

Cells were grown up under high  $\text{CO}_2$  conditions in 4 litre batches, soluble extract prepared as described in section 2.16.2 and a two stage purification process carried out on this extract involving high performance liquid chromatography (HPLC) and diethylaminoethyl-cellulose chromatography (DEAE).

##### 5.2.2.1 High performance liquid chromatography

This was performed with an LKB BROMMA system. A 60 cm TosohHaas gel filtration column (G3000SW) was used to fractionate the extract. The extract was loaded via a 2 ml sample loop at a flow rate of  $5 \text{ ml/min}^{-1}$ .

The UV detector was set at 1 absorbance unit, and 3 ml fractions were collected. These were then subjected to *in vitro* kinase assays, and relevant fractions bulked, and frozen at  $-70^{\circ}\text{C}$  until required for further analysis.

##### 5.2.2.2 Diethylaminoethyl-cellulose chromatography

HPLC fractions identified as containing proteins which were radiolabelled following *in vitro* kinase experiments, were subjected to further *in vitro* kinase reactions using 5x as much of each component of the kinase reaction (see 2.16.2), total volume 0.25 ml which was then loaded onto a 20 ml DEAE column. The column was packed with Trisacryl M (LKB), equilibrated in 2x



kinase buffer (pH 8.5). 30 ml of 2x kinase buffer was run through the system, then the radiolabelled HPLC fraction was loaded on top of the DEAE column. 10 ml of 2x kinase buffer (pH 8.5) was then run through the system, followed by a 25 mM - 0.5 M stepped salt gradient (NaCl in kinase buffer). 1 ml fractions were collected, the radioactivity in each measured in a scintillation counter (LKB), and appropriate fractions analysed by SDS-PAGE.

### 5.3 Results and discussion

#### 5.3.1 In vivo phosphorylation

##### 5.3.1.1 In vivo phosphorylation in *Synechocystis* PCC6803 continuously labelled with $^{32}\text{P}$ -orthophosphate during growth under different carbon regimes

Three 100 ml cultures of *Synechocystis* PCC6803 were grown to early logarithmic phase under low and high  $\text{CO}_2$  and photoheterotrophic conditions (see Section 2.5), radioactive  $^{32}\text{P}$ -orthophosphate (10  $\mu\text{Ci/ml}$ ) added to each culture, and at regular time intervals, 4 ml aliquots were removed and frozen.

A culture volume of at least double the sum total of sample volumes to be removed was used, to minimize any perturbations of the culture by the introduction of a large gas space and to help prevent an abnormally high apparent light intensity per cell due to an excessive decrease in the length of the light path through the culture.

The 4 ml samples were then defrosted when required, subjected to a BioRad microassay (see Section 2.14.1), and equal amounts of protein (usually 100  $\mu\text{g}$ ) loaded on each track.

Initially all radiolabelled samples were treated with 20  $\mu\text{g/ml}^{-1}$  Dnase I and RNase A for 30 minutes prior to loading the gel, however SDS-PAGE followed by autoradiography resulted in autoradiographs with a very high background (not shown) and so this method was abandoned in favour of hot TCA treatment, which was found to be just as effective without the DNase I and RNase

A pretreatment (see Section 2.16.3).

SDS-PAGE analysis and autoradiography (24hr exposure) of continuously labelled cells of *Synechocystis* PCC6803 grown under high CO<sub>2</sub>, low CO<sub>2</sub> and heterotrophic conditions, revealed a number of polypeptides which were subject to a phosphate-containing modification (see Figure 5.3). Mr estimations were carried out on the phosphopolypeptides by the preparation of a calibration curve using Pharmacia low molecular weight standards (see 2.15.2.1) and plotting log<sub>10</sub> Mr against relative mobility.

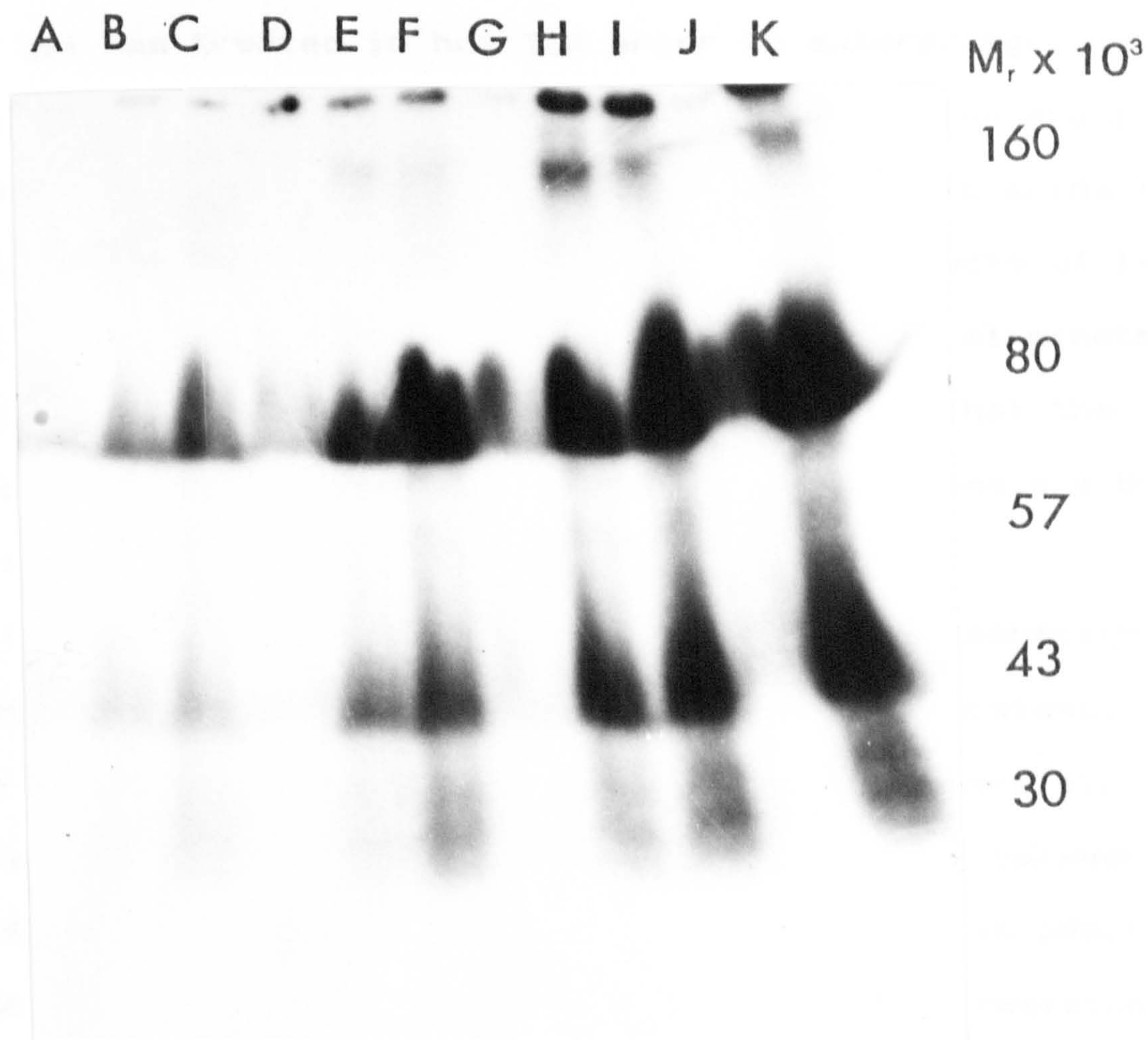
A number of phosphopolypeptides appear over the 8 hour time course including one common to all three different carbon regimes, with an Mr value of 80,000. Of the other phosphopolypeptides that appeared, none were present in cells grown under low CO<sub>2</sub> conditions, but appeared to be common to both high CO<sub>2</sub> and heterotrophic conditions. Along with the Mr 80,000 phosphopolypeptide, a phosphopolypeptide of Mr 43,000 represents one of the two major phosphopolypeptides. Of the remaining phosphopolypeptides there appears to be a doublet (or possibly a triplet) of around Mr 30,000 and possibly one of around Mr 57,000 appearing towards the end of the time course under both of these growth conditions.

One other phosphopolypeptide also appears under high CO<sub>2</sub> and heterotrophic growth conditions, but not low CO<sub>2</sub> growth conditions. This polypeptide has a high molecular weight, and it's Mr value can only be estimated, since it is outside the range of the molecular weight markers used. However by



Figure 5.3 : In vivo phosphorylation in *Synechocystis* PCC6803

Autoradiograph of *Synechocystis* PCC6803 continuously labelled with  $^{32}\text{P}$ -orthophosphate under different carbon regimes. Tracks A,D,G and J represent samples from low  $\text{CO}_2$ -grown cultures, labelled for 2,4,8 and 12 hours respectively. Tracks B,E,H and K represent samples from high  $\text{CO}_2$ -grown cultures, labelled for 2,4,8 and 12 hours respectively. Tracks C,F and I represent samples from heterotrophic cultures, labelled for 2,4 and 8 hours respectively.



extrapolating the graph of  $\log_{10}$  Mr value against relative mobility, an estimated Mr of 160,000 was obtained.

It is interesting to note that with the exception of the phosphopolypeptide of Mr 80,000, the rest of the phosphopolypeptides are common to the carbon regimes in which the rate of Ci uptake is severely reduced in comparison with low CO<sub>2</sub> grown cells (see Section 3.3.3.5 and Figure 3.9), even though the cells mode of metabolism is photoautotrophic under one of these carbon regimes and photoheterotrophic under the other.

This gel was treated in hot TCA prior to autoradiography, (a standard technique used in protein phosphorylation studies) (Bhorjee and Pederson, 1976) to eliminate nucleic acids and polyphosphates, which are two of the major products of labelling with <sup>32</sup>P-orthophosphate *in vivo*. In addition it eliminates acylphosphates and amidophosphates, suggesting that the phosphoproteins present are either O-phosphomonoesters or else the result of ADP-ribosylation.

All of the bands were eliminated by alkaline phosphatase treatment (200 units), strong evidence for the presence of *in vivo* ester-linked phosphopolypeptides, and all were also stable in neutral hydroxylamine (50 mM), which degrades between 70-90 % of ADP-ribosyl protein linkages (Burzio, 1982). In addition those of Mr 80,000, 57,000 and 43,000 also were resistant to hot alkali treatment, in which ADP-ribosyl and phosphoserine and phosphothreonine protein linkages are labile, suggesting they possess phosphotyrosine protein linkages whilst those



phosphopolypeptides around Mr 30,000 probably possess phosphoserine and phosphothreonine protein linkages (data not shown).

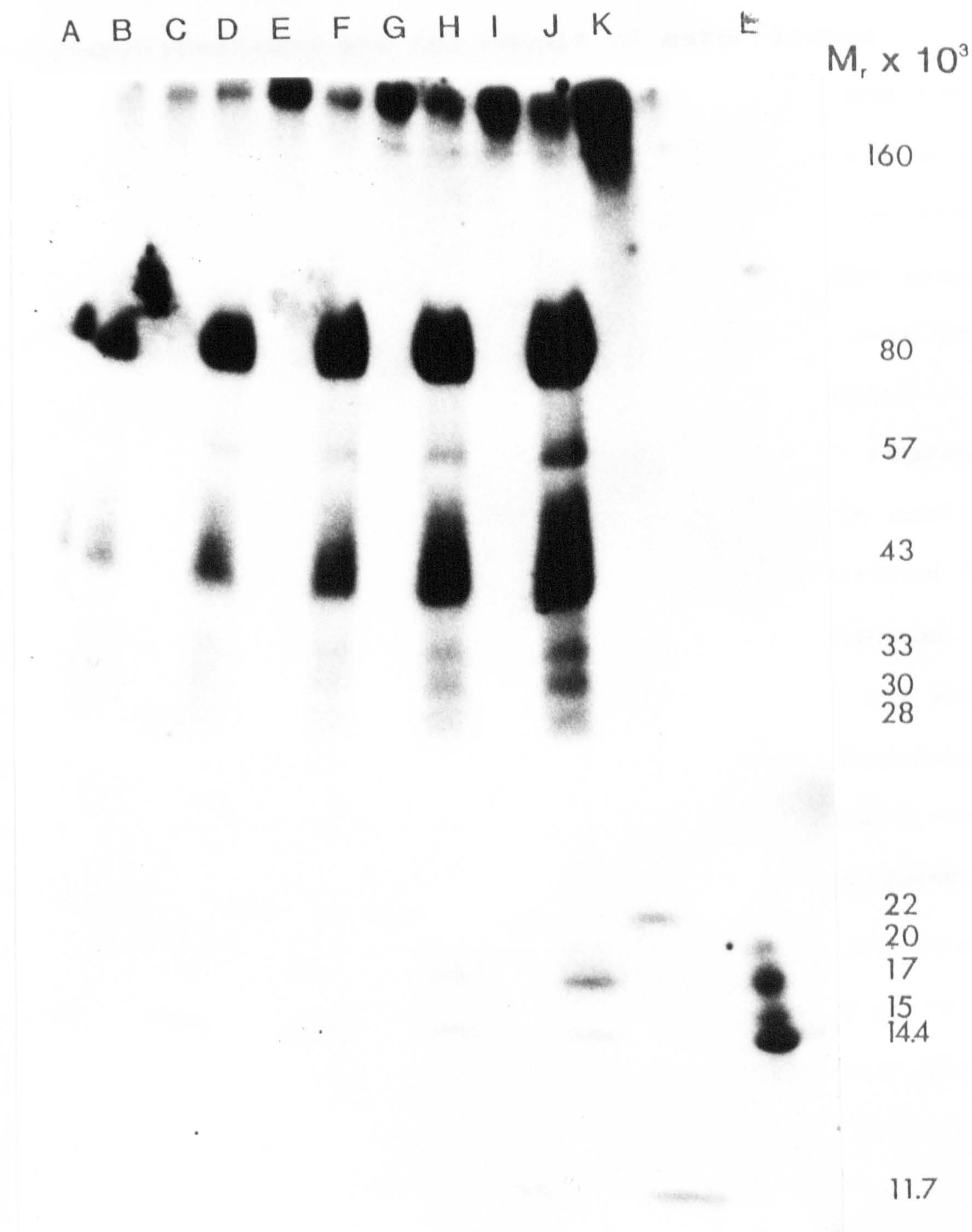
Figure 5.4 shows the  $^{32}\text{P}$ -orthophosphate incorporation into cells of *Synechocystis* PCC6803 and *Synechococcus* PCC7942 continuously labelled under low  $\text{CO}_2$  conditions and then transferred to high  $\text{CO}_2$  conditions for the duration of the time course. This autoradiograph shows more clearly the appearance of a number of phosphopolypeptides as a result of the switch from low to high  $\text{CO}_2$  growth conditions in *Synechocystis* PCC6803. As with Fig. 5.3, the phosphopolypeptide Mr 80,000 is again present under both low and high  $\text{CO}_2$  growth conditions, however phosphorylation was greatly stimulated under high  $\text{CO}_2$  conditions. Since the time course of 12 hours represents about one doubling time for the organism, and each track has an equal protein loading, it is clear that phosphorylation increases significantly more than can be accounted for by growth of the organism.

The high molecular weight phosphopolypeptide Mr 160,000 is again present, as is the faint band seen at Mr 57,000 in Fig 5.3. The band seen at Mr 43,000 could in fact represent more than one phosphopolypeptide, since the intensity of the autoradiographed band increases above Mr 43,000, suggesting that another phosphopolypeptide with a slightly higher Mr is present. There does indeed appear to be a phosphopolypeptide triplet around Mr 30,000, however the smallest of these, of Mr 28,000 has much less  $^{32}\text{P}$  phosphate incorporation than the other two phosphopolypeptides of Mr 30,000 and Mr 33,000.



Figure 5.4 : In vivo phosphorylation in *Synechocystis* PCC6803 and *Synechococcus* PCC7942

Autoradiograph showing time course of  $^{32}\text{P}$ -phosphate accumulation in *Synechocystis* PCC6803 and *Synechococcus* PCC7942 transferred from low to high  $\text{CO}_2$  growth conditions. Tracks A,B,D,F,H and J represent the time course for *Synechocystis* PCC6803, labelled for 0,1,2,4,8 and 12 hours respectively. Tracks C,E,G,I and K represent the time course for *Synechococcus* PCC7942, labelled for 1,2,4,8 and 12 hours respectively. Track L is an in vitro labelled sample from a high  $\text{CO}_2$ -grown, cell free extract of *Synechocystis* PCC6803.





As well as these phosphopolypeptides, which were also seen in Fig. 5.3, two low molecular weight phosphopolypeptides of Mr 17,000 and 14,400 also appeared on the switch to high CO<sub>2</sub> growth conditions. These two low molecular weight phosphopolypeptides also appeared in track L, which represents an *in vitro* labelling experiment using a high CO<sub>2</sub> grown cell homogenate of *Synechocystis* PCC6803. Since this procedure used gamma labelled ATP, it would seem very likely that these two phosphopolypeptides are the result of ester-linked phosphorylation as opposed to ADP-ribosylation, and the fact that they were degraded by alkaline phosphatase and snake venom phosphodiesterase (see Fig. 5.12) would seem to confirm this. Two more phosphopolypeptides, Mr 20,000 and 15,000 also appeared during the *in vitro* labelling experiment, which were not seen in *in vivo* labelled cells grown under any conditions.

The remaining tracks in Fig. 5.4 (C,E,G,I and K) represent the time course of <sup>32</sup>P-orthophosphate accumulation in continuously radiolabelled cells of *Synechococcus* PCC7942 switched from low to high CO<sub>2</sub> growth conditions. One phosphopolypeptide, Mr 22,000, can be seen to appear eight hours after the switch in CO<sub>2</sub> regimes. Another low molecular weight phosphopolypeptide can also be seen near the base of the gel. This appears over the same time scale as the Mr 22,000 polypeptide and seems to have a similar level of <sup>32</sup>P-orthophosphate incorporation. Since it lies outside the range of the molecular weight markers, its Mr can only be estimated by extrapolation of the calibration curve, which gave an estimated Mr of 11,500. The only other

phosphopolypeptide seen in *Synechococcus* PCC7942 appeared to have a similar molecular weight as the high molecular weight phosphopolypeptide seen in *Synechocystis* PCC6803 during growth under heterotrophic and high CO<sub>2</sub> conditions, which from Mr estimations appeared to be around Mr 160,000.

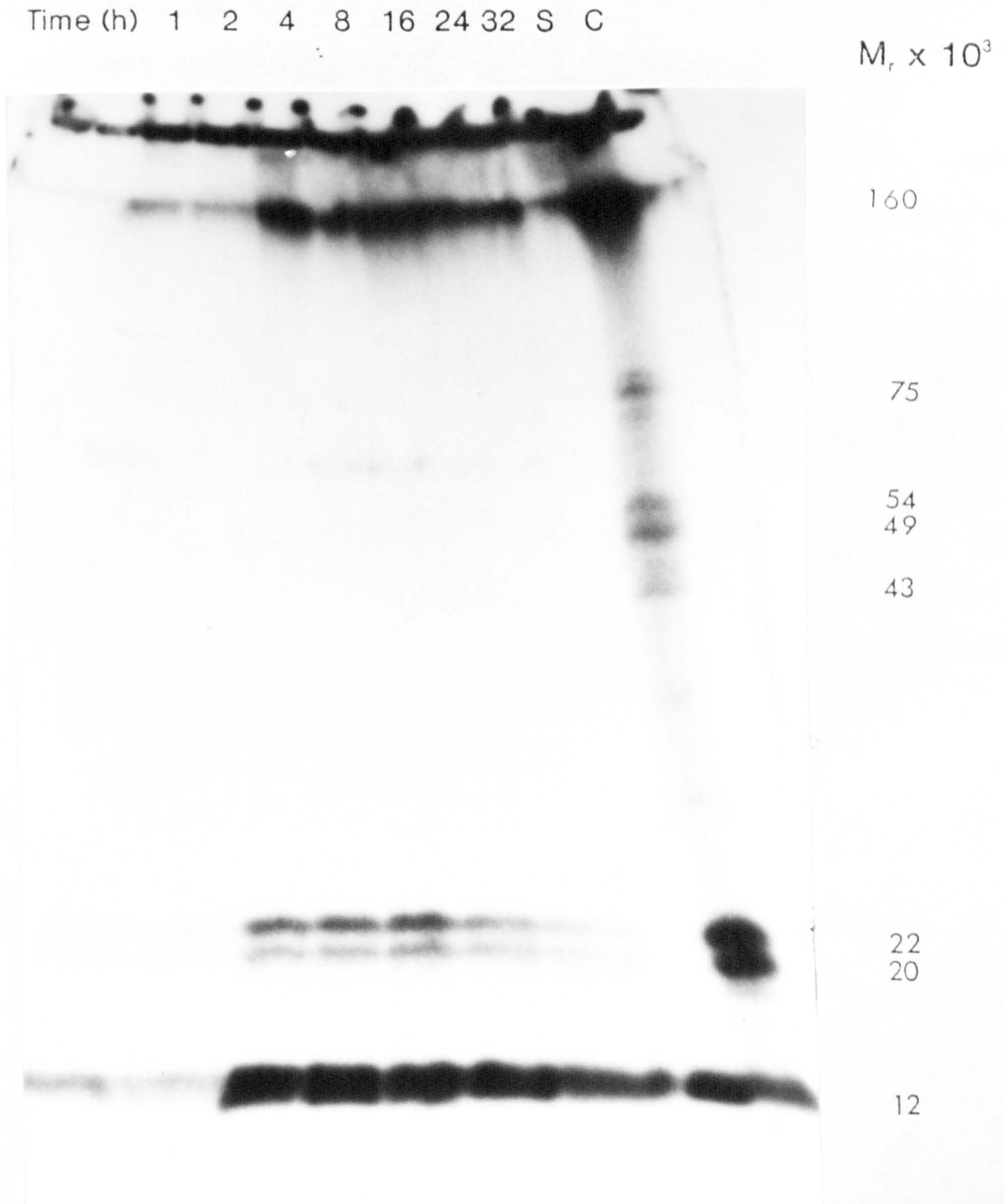
#### 5.3.1.2 In vivo phosphorylation in *Synechococcus* PCC7942

As radioisotope incorporation was much greater in *Synechocystis* PCC6803 than that seen in *Synechococcus* PCC7942, it was decided to run a gel using just samples of *Synechococcus* PCC7942, to see if any more phosphorylated proteins were present which might have been "masked" by the high amounts of incorporation seen in *Synechocystis* PCC6803. Fig. 5.5 shows the *in vivo* phosphorylation pattern of *Synechococcus* PCC7942 radiolabelled for 16 hours under low CO<sub>2</sub> and then switched to high CO<sub>2</sub> over a 32 hr time course. As in *Synechocystis* PCC6803, there appears to be very limited protein phosphorylation in low CO<sub>2</sub>-grown cells. The appearance of phosphoproteins started four hours after the shift to high CO<sub>2</sub> growth conditions, when four major phosphopolypeptides appear at Mr 12,000, 20,000, 22,000 and 160,000. Allowing for irregularities in the migration of proteins during SDS-PAGE, the phosphopolypeptides Mr 22,000 and Mr 12,000 are probably the same phosphopolypeptides as those seen in Fig. 5.4. A faint band, representing a phosphopolypeptide of Mr 60,000 was also seen in the 8 and 16 hour samples. Radioisotope incorporation appeared to diminish 16 hrs after the shift in growth regimes, however this is probably



Figure 5.5 : In vivo phosphorylation in *Synechococcus* PCC7942

Autoradiograph showing time course of  $^{32}\text{P}$ -phosphate accumulation in *Synechococcus* PCC7942 transferred from low to high  $\text{CO}_2$ -growth conditions. S, soluble fraction (8 hours). C, crude membrane fraction (8 hours).



due to the turnover of these proteins during cell metabolism, and was also seen in *Synechocystis* PCC6803 (data not shown). The last two tracks on the autoradiograph represent soluble (S) and crude membrane (C) fractions prepared from the remaining cells in the T=8 hr sample. These were centrifuged (5000 rpm, 5 min), resuspended in 5 ml 10 mM Tes-NaOH buffer (pH 7.0), and the cells disrupted by two passages through a French pressure cell. Cell debris was removed by centrifugation at 5,000 rpm for 5 min and the supernatant centrifuged in an MSE 10 x 10 centrifuge for 1 hr at 60,000 rpm. The pellet produced was used as a crude membrane sample whilst the supernatant was used as a soluble fraction. From this crude separation it would appear that the phosphopolypeptides Mr 160,000, 22,000 and 20,000 are exclusive to the membrane fraction of the cell, whilst the phosphopolypeptide Mr 12,000 is common to both. In addition, a number of other phosphopolypeptides were also present in the membrane fraction which did not appear in the time course. These appeared at Mr 54,000, Mr 49,000 and Mr 43,000 and migrating around Mr 75,000 were a trio of phosphopolypeptides. These must all represent proteins with too little  $^{32}\text{P}$ -orthophosphate incorporation to show in the 4 ml samples taken during the time course. The Mr 60,000 phosphopolypeptide did not show in either the soluble or membrane fractions.

If we compare the results from both *Synechococcus* PCC7942 and *Synechocystis* PCC6803, it can be seen that in both organisms there is very little protein phosphorylation under low  $\text{CO}_2$  conditions, where Ci is limiting and the inorganic carbon



concentrating mechanism is in operation, and yet with a switch to growth conditions where this mechanism is either not present, or operating at much reduced levels, a number of phosphopolypeptides appear. This is not that surprising, it is likely that similar control mechanisms exist in the two organisms, and phosphorylation/dephosphorylation, as already discussed in the introduction to this chapter, is a common mechanism used to regulate a cells metabolic and energy requirements

If the time course over which these phosphopolypeptides appear is examined, it can be seen that in *Synechocystis* PCC6803 the appearance of the phosphopolypeptides follows a similar time course to the decay of the  $C_i$  concentrating mechanism (see Fig. 3.10), making it tempting to speculate that the phosphorylation of these proteins may be related to the inactivation of the process, however there is no direct evidence for this, and this could not be proven until the proteins have been identified. The protein phosphorylation seen could just as easily be responsible for changes in the activity of various proteins in metabolic pathways (activated/deactivated when there is a change in carbon levels in the cell), or for controlling energy fluxes in the cell, which will also vary under the different carbon regimes (a number of phosphopolypeptides have been discovered in cyanobacteria which regulate state I - state II transitions as discussed in section 5.1.4.4.).

As  $^{32}\text{P}$ -radioisotope incorporation was greater in *Synechocystis* PCC6803 than in *Synechococcus* PCC7942, allowing a much quicker



visualisation of autoradiographs, and because a number of similar phosphopolypeptides appeared following the switch from both low  $\text{CO}_2$  to high  $\text{CO}_2$  and heterotrophic conditions it was decided to try and further characterize the regulation of these phosphopolypeptides in this organism.

#### 5.3.1.3 Further characterization of *in vivo* phosphorylation in *Synechocystis* PCC6803

It is known that induction of the Ci concentrating mechanism requires protein synthesis (Marcus et al., 1982; Omata and Ogawa, 1986) and although the changes in phosphorylation pattern seen were under conditions where the Ci uptake mechanism is greatly reduced or absent, it was decided to establish whether any of the phosphoproteins were synthesized *de novo*, or if they were simply existing proteins whose activity was regulated by phosphorylation. A low  $\text{CO}_2$ -grown culture (250 ml) was grown to early logarithmic phase under continuous labelling conditions and at  $T=0$ , separated into two equal cultures and incubated under high  $\text{CO}_2$  conditions. To one of these parallel cultures 100 ug/ml chloramphenicol was added, whilst the other was left untreated. At regular time intervals 4 ml samples were removed from both cultures and frozen until required. Figure 5.6 shows the autoradiograph obtained from samples of the above experiment run on SDS-PAGE (100 ug protein/track). Although the amount of radioisotope incorporation is lower in the culture to which chloramphenicol was added, the same pattern of bands occurs.

This suggests that none of the phosphopolypeptides (or kinases) is the result of *de novo* synthesis. The lower amount of radioisotope incorporation seen in the chloramphenicol treated culture is probably due to the general effects of this substance on the cells metabolism, resulting in a much slower metabolic rate for all cell processes, which will have a knock-on effect on radioisotope incorporation, because the level of radioisotope incorporation increases at each time point whereas in the culture with no chloramphenicol added, radioisotope incorporation reaches a peak after 12 hours, and then declines gradually.

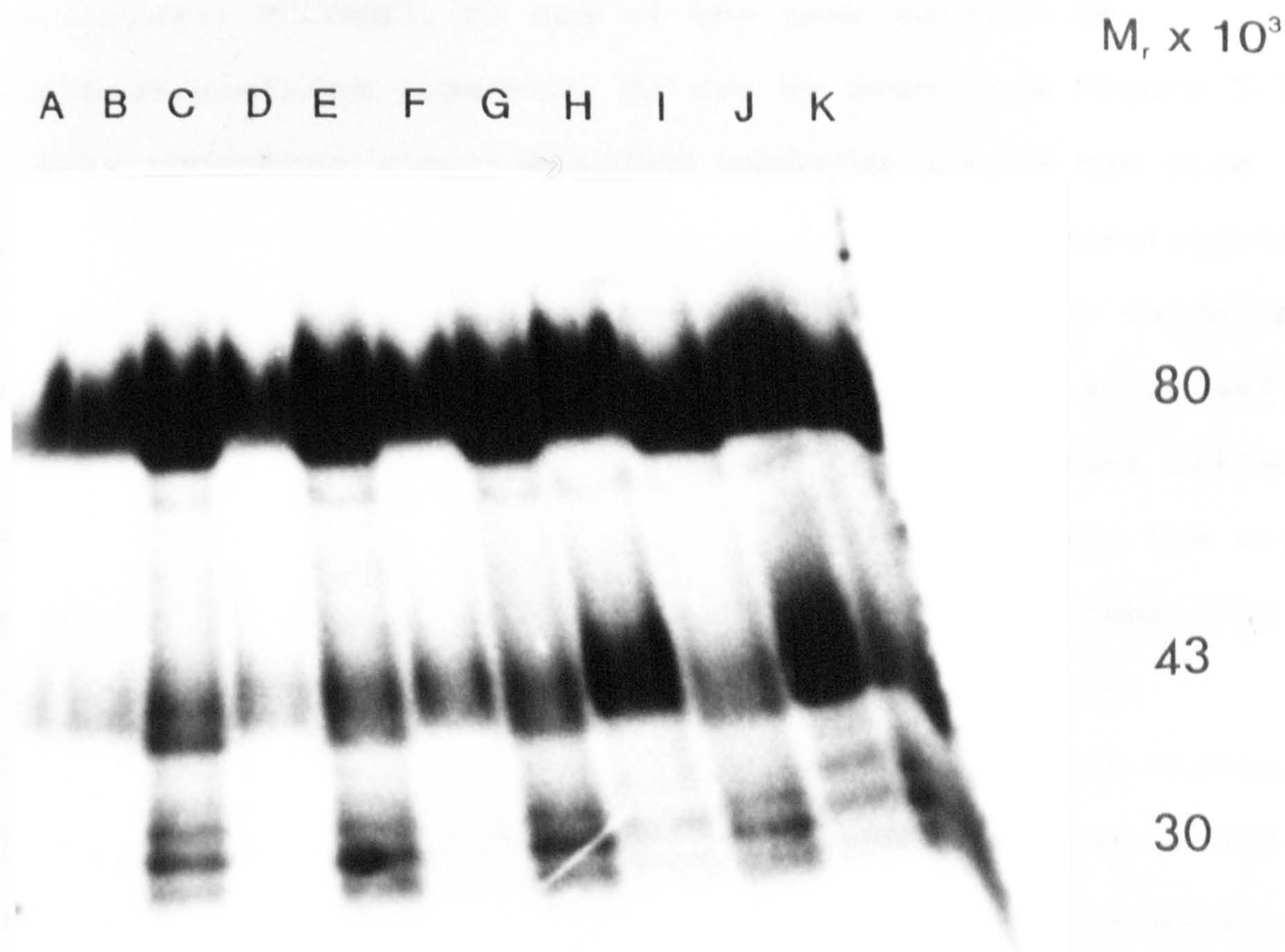
A mechanism can be hypothesized whereby transfer to limiting Ci conditions leads to the generation of a Ci concentrating mechanism, and upon transfer to conditions of nutrient excess, certain components of the mechanism are phosphorylated to inactivate the mechanism on a short term basis, with the actual components of the mechanism that were synthesized upon transfer to Ci limiting conditions being broken down and turned-over, in the long term.

Addition of 1 mM of the structural analogue, 3-O-methyl-glucose (OMG) to low CO<sub>2</sub>-grown cultures of *Synechocystis* PCC6803 has been shown to have no effect on the Ci uptake system exhibited by these cells (see Fig 3.9). In the same manner, addition of OMG (1 mM) to a low CO<sub>2</sub>-grown culture continuously labelled with <sup>32</sup>P had no effect on the *in vivo* phosphorylation pattern in this organism, and as in low CO<sub>2</sub>-grown cultures the only



Figure 5.6 : Effect of chloramphenicol on *in vivo* protein phosphorylation in *Synechocystis* PCC6803

Autoradiograph showing time course of  $^{32}\text{P}$ -phosphate accumulation in *Synechocystis* PCC6803 transferred from low to high  $\text{CO}_2$  growth conditions. Track A, low  $\text{CO}_2$  labelled cells ( $T=0$ ). Tracks B,D,F,H and J represent the time course for *Synechocystis* PCC6803 + chloramphenicol (100  $\mu\text{g}/\text{ml}$ ), labelled for 4,8,12,18 and 24 hours respectively. Tracks C,E,G,I and K represent the time course for *Synechocystis* PCC6803, labelled for 4,8,12,18 and 24 hours respectively.

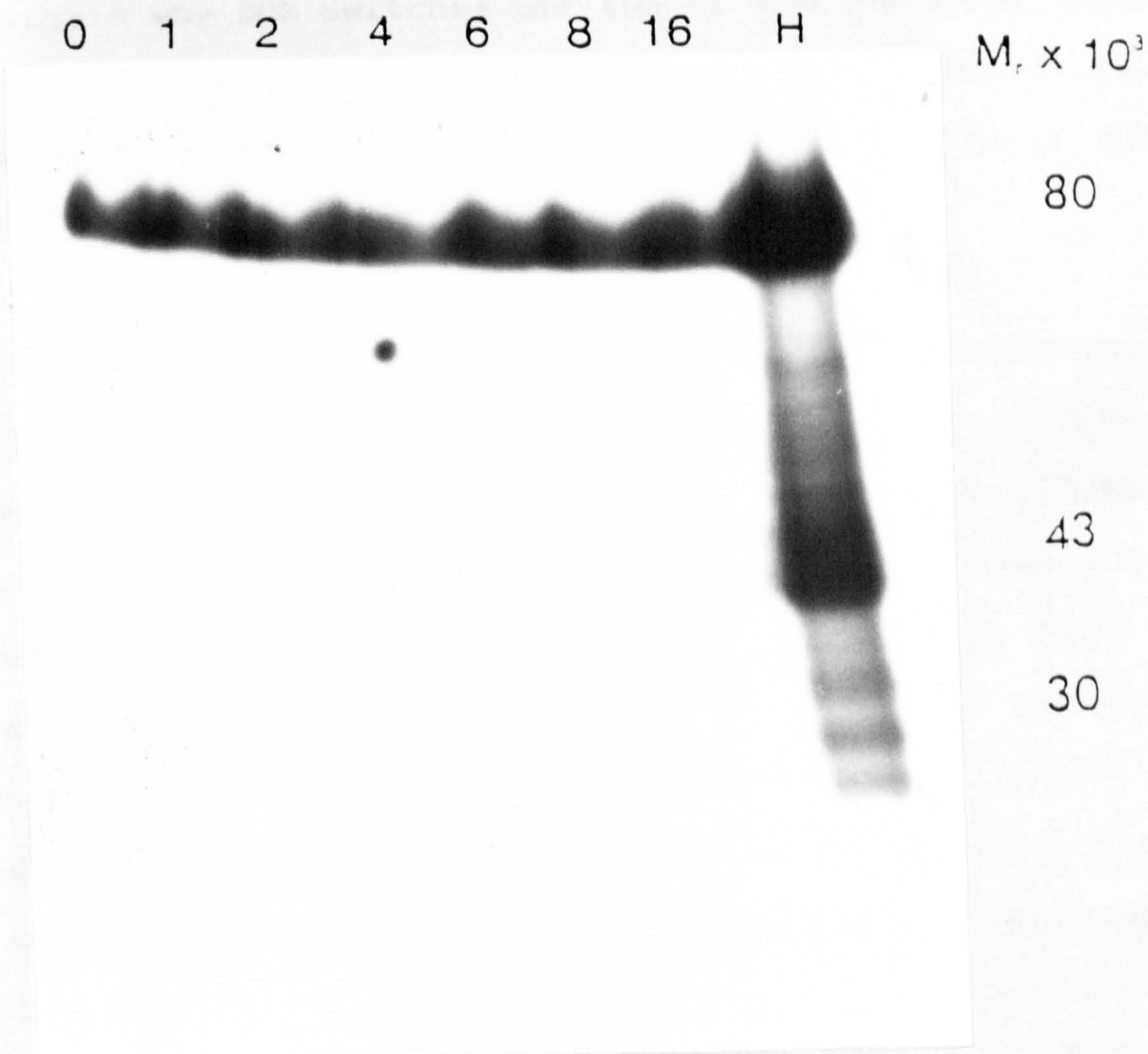




phosphopolypeptide present was at Mr 80,000 (data not shown). It has also been shown in this study that addition of 1 mM of the structural analogue of glucose, 2-Deoxy-D-glucose (DOG), to low CO<sub>2</sub>-grown cultures of *Synechocystis* PCC6803 caused a decay in the Ci uptake system exhibited by these cells (see Fig 3.9). As both glucose and bicarbonate caused both a decay in the Ci concentrating mechanism and also an increase in the number of phosphopolypeptides visualised on SDS-PAGE, DOG (1 mM) was added to <sup>32</sup>P-orthophosphate labelled low CO<sub>2</sub>-grown cultures of *Synechocystis* PCC6803, to see if the same pattern of phosphopolypeptides appeared. As can be seen from Figure 5.7, the only phosphopolypeptide which appeared during the time course when DOG was added was the Mr 80,000 phosphopolypeptide. As both DOG and OMG are non-metabolizable, are both taken up by *Synechocystis* PCC6803, do not alter the phosphorylation pattern of low CO<sub>2</sub>-grown *Synechocystis* PCC6803, and yet exert different effects on the Ci uptake mechanism, it suggests that the signal for switching off the Ci uptake mechanism is different from that which results in the phosphorylation pattern seen in photoheterotrophically grown cells. As both of these analogues are non-metabolizable, it is likely that the changes in the pattern of phosphorylation seen upon the addition of glucose occurs upon the metabolism of the substrate. In a similar vein, as both analogues can enter *Synechocystis* PCC6803, it is unlikely that the Ci concentrating mechanism is switched off upon transport across the membrane by the glucose transport system. As the fate of glucose upon entering the cell is

Figure 5.7 : Effect of 2-deoxy-D-glucose on in vivo protein phosphorylation in *Synechocystis* PCC6803

Autoradiograph showing time course of  $^{32}\text{P}$ -phosphate accumulation following addition of 2-deoxy-D-glucose (1 mM) to low  $\text{CO}_2$ -grown *Synechocystis* PCC6803. H, T=8 hour sample from a low to high  $\text{CO}_2$  in vivo shift experiment





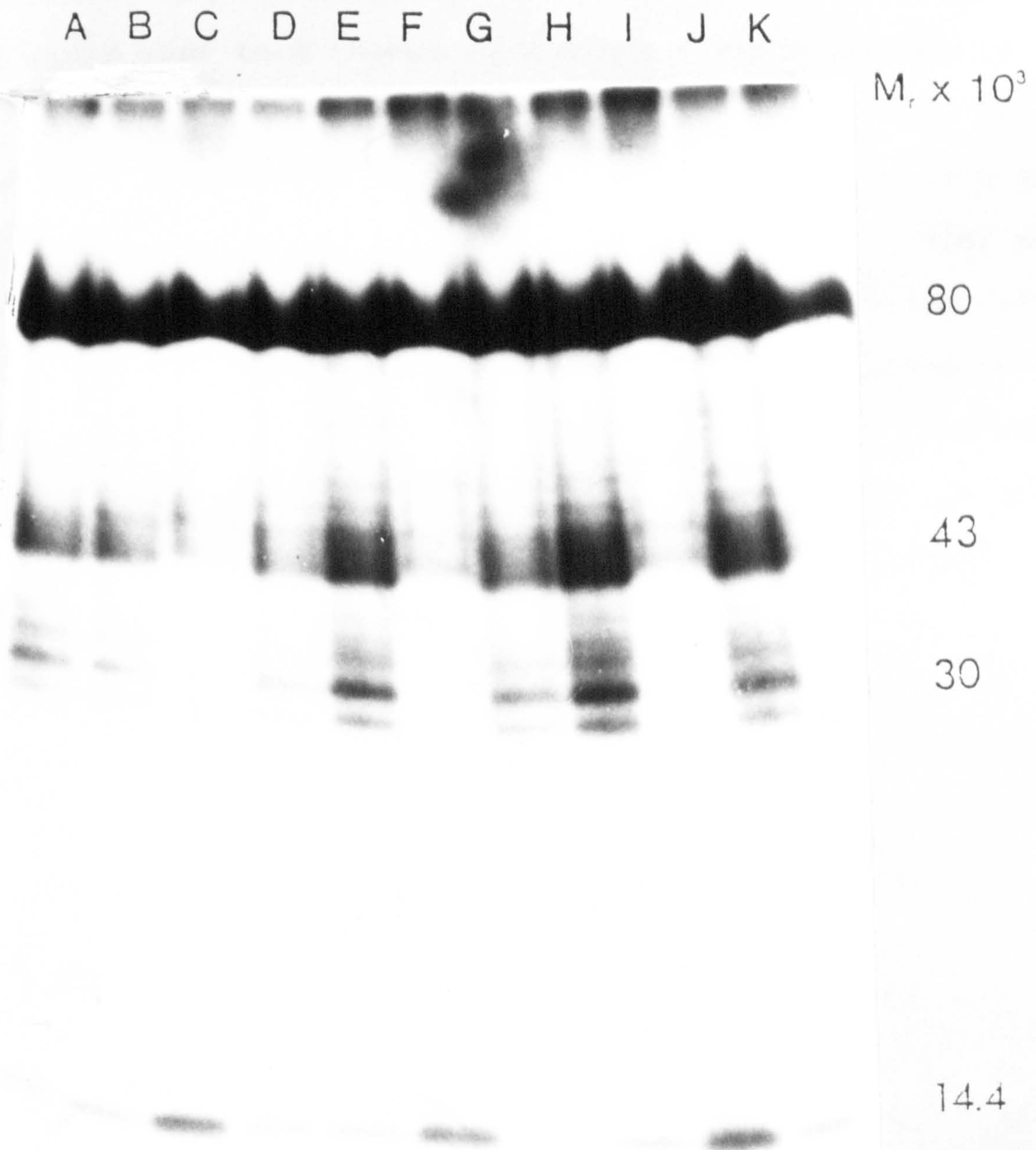
phosphorylation by ATP, catalysed by hexokinase, to form glucose-6-phosphate, it is likely that glucose and DOG are both phosphorylated by hexokinase, but OMG because of its side chain addition, cannot enter the active site, and so remains unphosphorylated. It can be hypothesized that a build-up of a pool, or in the case of DOG, an apparent pool, of glucose-6-phosphate leads to the  $C_i$  uptake mechanism being switched off. If this hypothesis proves to be true, it would explain why DOG switches off the  $C_i$  concentrating mechanism, but does not cause an alteration in the phosphorylation pattern seen, because obviously a phosphorylated version of DOG would still be unmetabolizable.

Fructose, although capable of supporting the photoheterotrophic growth of many cyanobacteria (see Rippka et al., 1979), is bactericidal for *Synechocystis* PCC6803 and its transport is competitively inhibited by glucose and OMG (Flores and Schmetterer, 1986; Joset et al., 1988). Fig 5.8 shows the time course of radioisotope incorporation following addition of glucose (tracks B,E and H), fructose (tracks C,F and I) and glucose and fructose (tracks D,G and J) to low  $CO_2$ -grown cells of *Synechocystis* PCC6803. This autoradiograph shows, due to the lower amounts of radioisotope incorporation that the very intense band at Mr 43,000 seen on previous autoradiographs of this organism (Fig. 5.3, 5.4, 5.6 and 5.7) is due to the presence of at least four phosphopolypeptides. If the actual pattern of phosphorylation between the three



Figure 5.8 : In vivo phosphorylation in *Synechocystis* PCC6803;  
The fructose effect

Autoradiograph showing time course of  $^{32}\text{P}$ -phosphate accumulation in *Synechocystis* PCC6803 transferred from low  $\text{CO}_2$  to different carbon regimes. Tracks B, E and H represent the addition of glucose (10 mM) for 4, 8 and 12 hours respectively. Tracks C, F and I represent the addition of fructose (10 mM) for 4, 8 and 12 hours respectively. Tracks D, G and J represent the addition of glucose + fructose (10 mM) for 4, 8 and 12 hours respectively. Track A, T=8 hour in vivo sample from a low  $\text{CO}_2$  to low  $\text{CO}_2$  + glucose (10 mM) shift experiment. Track K, T=8 hour in vivo low  $\text{CO}_2$ -grown sample.





different growth conditions are compared it can be seen that the addition of fructose (10 mM) to *Synechocystis* PCC6803 results in the inhibition of phosphorylation of the triplet of polypeptides around Mr 30,000, and stimulation of phosphorylation of a polypeptide Mr 14,400 in comparison to the addition of glucose. The phosphorylation pattern is not however the same as that seen in low CO<sub>2</sub>-grown or OMG treated cells, suggesting that the fructose has been metabolized once transported into the cell and Flores and Schmetterer (1986) thought that it was some metabolite of fructose, rather than fructose which was responsible for the bactericidal effect. It can only be speculated that this change in phosphorylation pattern is responsible or partly responsible for the bactericidal effect of fructose due to the switch on and off of various enzymes in the metabolic pathways. In addition both Joset et al. (1988) and Zhang et al. (1989) have reported that the bactericidal effect of fructose can be relieved by the addition of glucose or OMG, demonstrating since OMG is non-metabolizable, that this relief is due to competition at the glucose permease. Addition of 10 mM glucose relieved the changes seen in the phosphorylation pattern, leading to the normal pattern seen in photoheterotrophically grown cells (tracks D,G and J), confirming that glucose had been preferentially taken up by the cell, and Joset et al. (1988) reported that fructose uptake in both *Synechocystis* PCC6803 and PCC6714 took place at a very low rate.

It is known that some of the proteins involved in the PTS system

are phosphorylated (see Dills et al., 1980), however, both Zhang et al. (1989) and Schmetterer (1990) have identified the glucose permease in *Synechocystis* 6803 as being homologous with a group of sugar transporters using non-phosphorylating processes.

It is well documented that the accumulation of  $C_i$  by cyanobacteria is dependent on the supply of photosynthetic energy and is reduced by the whole-chain electron-transport inhibitor DCMU (see Badger, 1987 and section 1.5.2). It was initially thought that photosynthetic reactions were linked to  $C_i$  transport through the synthesis of ATP by PSI, however it now seems the  $C_i$  concentrating mechanism is driven by ATP synthesis from PSI with PSII providing reducing equivalents needed for activation of transport (Kaplan et al., 1987). Phosphorylation is known to be involved in the regulation of excitation energy distribution through state I - state II transitions in green plants (Kyle et al., 1984), and Allen and Holmes (1986) proposed a general model for the regulation of photosynthetic unit function by protein phosphorylation, which can be applied to the PS II of cyanobacteria as well as eukaryotes. Recent work in plants suggests that protein phosphorylation may be responsible for modulating responses to  $C_i$  concentration. Stromal protein phosphorylation in isolated chloroplasts of *Spinacia oleracea* increased in response to  $CO_2$  fixation (Foyer, 1985), and in the same organism it has been demonstrated that in both intact chloroplasts and isolated thylakoid membranes the 25 KDa polypeptide of LHC II is under the regulation of



bicarbonate, which also had a negative influence on the phosphorylation of the 9 KDa polypeptide of PS II (Sundby et al., 1989). The photosynthetic apparatus of cyanobacteria includes an electron transport chain very similar to that of plants. The PSI antenna of cyanobacteria is composed only of chlorophyll a, whilst the phycobilisome (PBS), an elaborate high molecular weight protein complex transmits energy to PSII (Tandeau de Marsac and Cohen-Bazire, 1977). The PBS consists of two protein classes, the biliproteins and the linker polypeptides. These linker polypeptides are responsible for the structural organisation and stability of the complex and allow directional transfer of the absorbed light energy (Tandeau de Marsac and Cohen-Bazire, 1977). Elmorjani et al. (1986) reported that the major polypeptides found in the PBS of *Synechocystis* PCC6803 were the same and had similar molecular masses as those seen in the other species so far analysed. These included three linker polypeptides of Mr 35,000, 33,000 and 27,000, which have molecular masses very similar to three of the phosphopolypeptides seen under high CO<sub>2</sub> and photoheterotrophic conditions. The control of energy fluxes through these linker polypeptides could be a mechanism by which the Ci concentrating mechanism is turned on and off, and as these polypeptides have been found in all cyanobacteria studied it could be a universal mechanism, with phosphorylation and dephosphorylation regulating these fluxes under conditions of substrate excess and limitation. To see if these phosphopolypeptides were indeed phycobilisome linker proteins, phycobilisomes were isolated from

photoheterotrophically and low and high CO<sub>2</sub> grown *Synechocystis* PCC6803 continuously labelled with <sup>32</sup>P-orthophosphate as detailed in section 5.2.1. The sucrose gradient obtained following this procedure, and the absorption spectra of the blue band showing the characteristic absorption maximum at 625 nm (see Yamanaka et al., 1978) are shown in Figure 5.9.

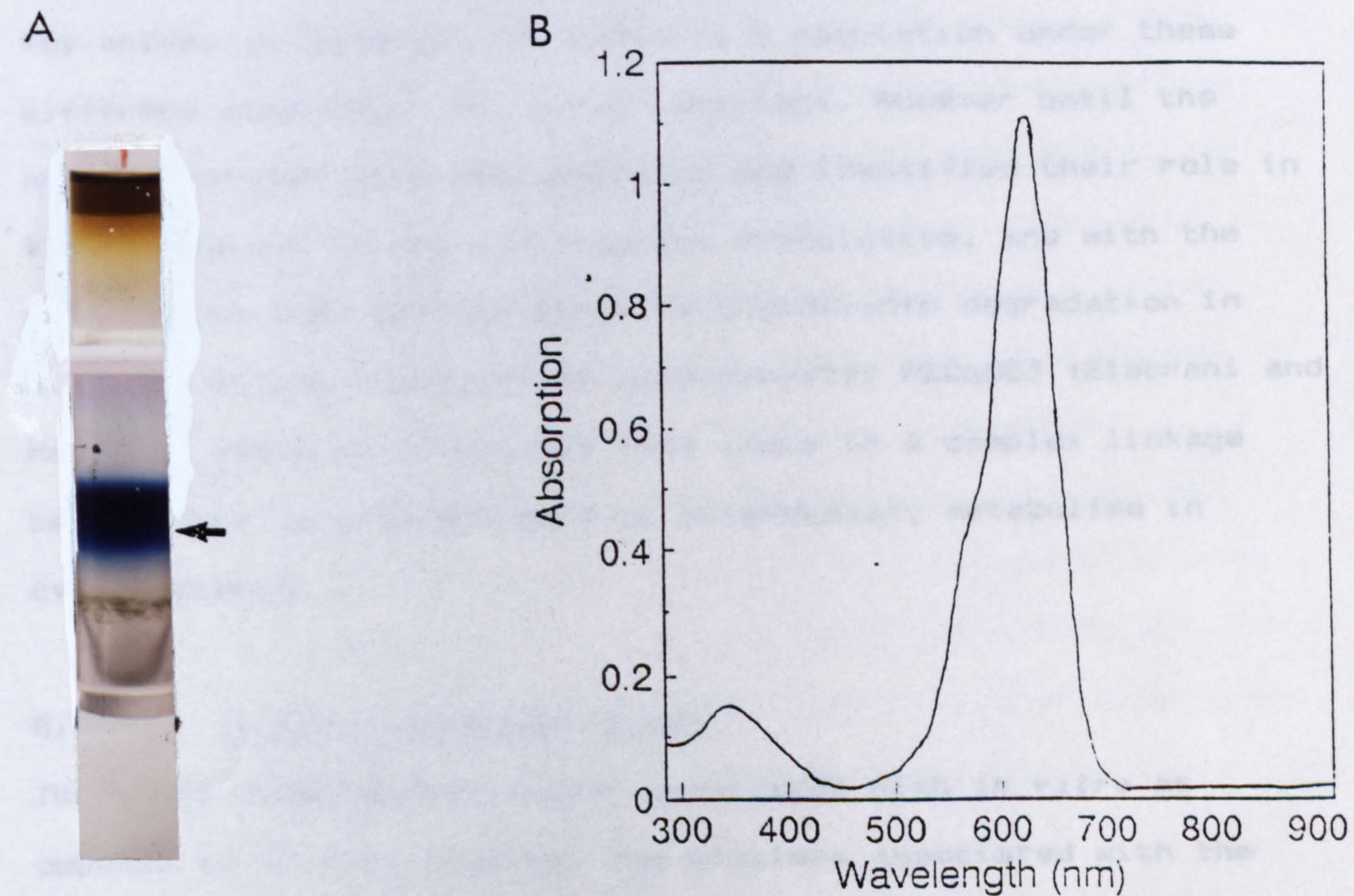
SDS-PAGE of the phycobilisome preparation, followed by autoradiography indicated that neither the triplet of phosphopolypeptides around Mr 30,000 nor any of the other phosphopolypeptides are phycobilisome associated proteins. *In vitro* labelling of purified phycobilisome preparations also indicated that no phosphopolypeptides are present in the phycobilisome (data not shown). If the phosphorylated proteins are involved in regulating energy fluxes in the cell, it is at a stage that apparently does not involve the phycobilisomes.

The phosphorylated proteins could also be involved in regulating the metabolic pathways involved in intermediary metabolism in this organism, which will vary depending on the nutrient levels supplied to the cell. Smith (1982) stated that since the machinery of photosynthesis and respiratory electron transport are constitutively expressed, control of the pathways operating in the light and dark must be exerted at the enzymic level. The absence of light is in itself a compulsory control, and it is envisaged that fine controls operate on the respiratory and photosynthetic electron transport chain (see Reinhold et al., 1987; Price and Badger, 1989a). It is well established that phosphorylation of isocitrate dehydrogenase in *E. coli* leads to



Figure 5.9 : Isolation of phycobilisomes from *Synechocystis* PCC6803

- A) Phycobilisome fraction (arrowed) from *Synechocystis* PCC6803 isolated by sucrose density gradient centrifugation.
- B) Absorption spectrum of isolated phycobilisome fraction.





it's complete inactivation (see section 5.1.4.1).

Under both high  $\text{CO}_2$  and photoheterotrophic growth conditions, the amount of utilizable substrate is greater than under low  $\text{CO}_2$  conditions and hence the intermediary metabolite levels are also probably higher and need to be tightly regulated.

Levels of RUBP are higher in high as opposed to low  $\text{Ci}$  grown *S. leopoliensis* (Mayo et al., 1989), and the enzyme ADP-glucose pyrophosphorylase is known to be regulated by the balance between 3-PGA and phosphate (Levi and Preiss, 1976), and as a key enzyme in glycogen synthesis it's regulation under these different conditions may prove important. However until the phosphoproteins have been isolated and identified their role in the metabolism of the cell remains speculative, and with the observation that glucose inhibits phycocyanin degradation in nitrogen starved cultures of *Synechocystis* PCC6803 (Elmorani and Herdman, 1987) it is obvious that there is a complex linkage between the various pathways of intermediary metabolism in cyanobacteria.

### 5.3.2 In vitro phosphorylation

There are numerous advantages associated with *in vitro* as opposed to *in vivo* studies. The problems associated with the incorporation of radiolabel into nucleic acids and phospholipids are eliminated, enabling much smaller amounts of radioisotope to be used, with corresponding safety advantages. Reaction conditions can be modified allowing factors modulating kinase and phosphatase activities to be studied and the ability to

perform a whole series of experiments on one previously prepared extract results in a level of consistency probably unobtainable *in vivo*.

The singular major disadvantage with *in vitro* studies is that the phosphopeptide patterns visualized rarely show much resemblance to those observed from *in vivo* experiments (see Manai and Cozzone, 1982, Turner, 1987). There are several possible explanations for this. All the results obtained *in vitro* could be artifacts, produced by non-specific phosphorylation of proteins normally compartmentalized away from the kinases, however this is unlikely, as prokaryotic protein kinases appear to be highly specific, unlike the generalized phosphorylation seen in eukaryotic kinases such as protein kinase C. As conditions used *in vitro* are non-physiological, this could lead to activation and inactivation of different kinases from those seen *in vivo*. There are also other possibilities, such as the loss of regulatory polypeptides during the extract fractionation procedure, the separation of kinase/phosphatase from their subject protein if they were closely linked *in vivo* and also the lack of further processing (e.g. proteolytic cleavage) which could take place *in vivo*.

#### 5.3.2.1 *In vitro* phosphorylation in *Synechocystis* PCC6803

*In vitro* phosphorylation was carried out as described in section 2.16.2. Intermediary metabolites were added at a concentration of 1 mM unless otherwise stated. Fig. 5.10 shows the *in vitro* protein phosphorylation pattern of cell-free extracts of high

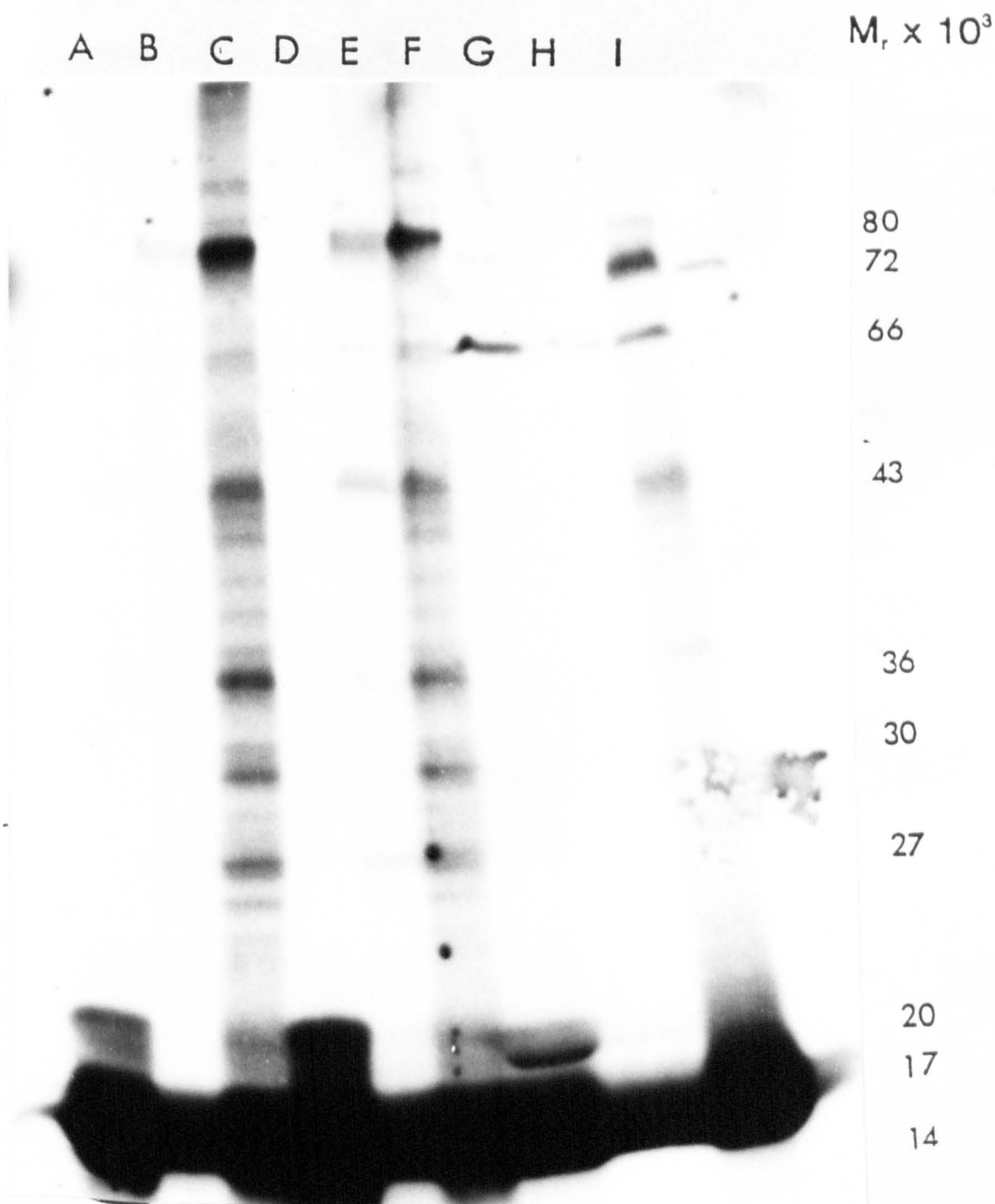
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Figure 5.10 : In vitro phosphorylation in *Synechocystis* PCC6803

Autoradiograph showing in vitro phosphorylation pattern of cell-free extracts from heterotrophic and high and low CO<sub>2</sub>-grown cultures of *Synechocystis* PCC6803.

Tracks A,D and G represent cell-free extracts from high CO<sub>2</sub>-grown cells. Tracks B,E and H represent cell-free extracts from heterotrophically-grown cells. Tracks C,F and I represent cell-free extracts from low CO<sub>2</sub>-grown cells. In tracks A,B and C the test substance was water, in tracks D,E and F the test substance was potassium ferricyanide (1 mM) and in tracks G,H and I the test substance was sodium dithionate (1 mM).





(H) and low (L) CO<sub>2</sub> and photoheterotrophically (HET) grown *Synechocystis* PCC6803. The majority of the phosphopolypeptides are present in cell free extracts from low CO<sub>2</sub>-grown cells (tracks c,f and i), which is not suprising since under these conditions *in vivo* only the Mr 80,000 protein is phosphorylated (see Fig. 5.3). The high CO<sub>2</sub> (tracks A,D and G) and extracts from heterotrophically grown cell do not, with the exception of polypeptide Mr 64,000 seen under reducing conditions in high CO<sub>2</sub>-grown cells (track G), have any high molecular weight polypeptides phosphorylated *in vitro*. Under these growth conditions most of the polypeptides are already phosphorylated *in vivo* (see Fig. 5.3). Common to all three growth conditions are two very intensely radiolabelled phosphopolypeptides of Mr 14,000 and Mr 15,000. Two other low molecular weight polypeptides were also phosphorylated *in vitro* in the low and high CO<sub>2</sub> extracts at Mr 17,000 and Mr 20,000. In comparison to the control tracks (A,B and C), addition of potassium ferricyanide to the high CO<sub>2</sub> extract, stimulated *in vitro* phosphorylation of these low molecular weight polypeptides, and in addition stimulated the phosphorylation of another polypeptide of Mr 13,000. In contrast, dithionate, a reducing agent, inhibited *in vitro* phosphorylation of the Mr 20,000 polypeptide and greatly reduced the amount of phosphorylation in the Mr 17,000 polypeptide. Dithionate also inhibited *in vitro* phosphorylation in the Mr 23,000, Mr 27,000, Mr 31,000, Mr 47,000 and Mr 80,000 polypeptides seen in low CO<sub>2</sub> extracts, but stimulated *in vitro* phosphorylation in three polypeptides Mr

64,000, Mr 70,000, and Mr 72,000.

Addition of sodium dithionate and potassium ferricyanide to the *in vitro* reaction mixture will affect the redox potential of the reaction mixture, probably through interactions with ferredoxin/thioredoxin. A number of enzymes involved in cyanobacterial metabolism are regulated by the ferredoxin/thioredoxin system, and it could provide a means whereby the enzymes of CO<sub>2</sub> fixation and carbohydrate degradation are modulated to meet the varying metabolic needs of the cell (see Rowell et al., 1988). The fact that dithionate and ferricyanide affected the *in vitro* phosphorylation state of various polypeptides means that the redox state of the cell is very important in determining phosphorylation state, and is probably responsible in part for controlling phosphorylation in the cell (see Harrison et al., 1991).

With regards to the similarities between *in vivo* and *in vitro* phosphorylation, it has already been shown (see Fig. 5.4) that two phosphopolypeptides Mr 17,000 and 14,400 appear to be common to both *in vitro* and *in vivo* labelling. The Mr 80,000 phosphopolypeptide and those at Mr 30,000 and Mr 27,000 (allowing for discrepancies between gels) also have similar molecular weights to phosphopolypeptides labelled *in vivo*. From Figure 5.10, it would appear that no other phosphopolypeptides found under *in vivo* labelling conditions appear during *in vitro* labelling, under the conditions employed. As already mentioned, the lack of resemblance of phosphopolypeptides under the different labelling conditions is not uncommon (see Manai and



Cozzone, 1982 and Turner, 1987).

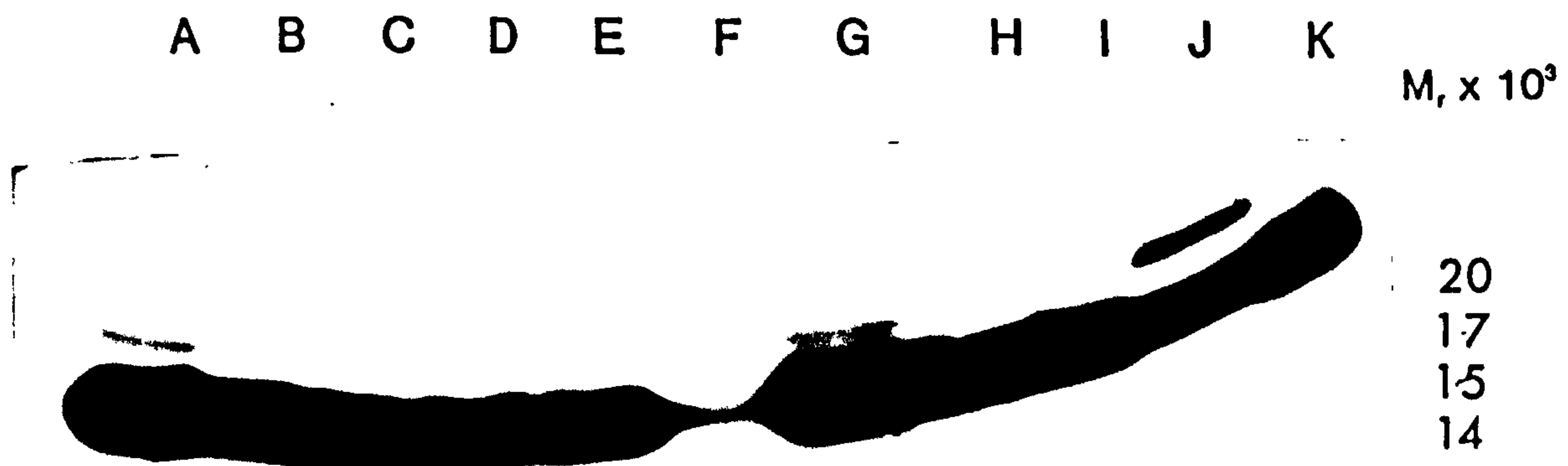
#### 5.3.2.2 In vitro phosphorylation in cell-free extracts of high $\text{CO}_2$ -grown *Synechocystis* PCC6803

Although the majority of polypeptides phosphorylated *in vitro* are found in low  $\text{CO}_2$ -grown cell-free extracts, it was decided to carry out further investigations on *in vitro* phosphorylation in *Synechocystis* PCC6803 using high  $\text{CO}_2$ -grown cell-free extracts, since with fewer phosphopolypeptides present it was hoped that further classification and crude purification of at least one of the polypeptides would be possible. Fig. 5.11 shows the effects of intermediary metabolites and other effectors on *in vitro* phosphorylation. Glucose,  $\text{NaHCO}_3$ , OMG, NADPH and NADH (tracks C,D,E,H and I) showed no signs of stimulation/inhibition of *in vitro* phosphorylation in comparison with the control (track K). The addition of ferricyanide, RUBP and NaCl (0.5 M), stimulated *in vitro* phosphorylation of the Mr 20,000 polypeptide, although addition of NaCl inhibited *in vitro* phosphorylation of the polypeptide Mr 17,000. Dithionate (track B) again inhibited *in vitro* phosphorylation of the Mr 20,000 polypeptide, but did not stimulate phosphorylation of any high Mr polypeptides. The test substance in track F was 1 mM ATP, and as expected the level of radioisotope incorporation was reduced due to competition from the unlabelled ATP.

As the acceptor molecule for  $\text{CO}_2$  fixation, catalyzed by RuBisCO, RUBP is a key component of the Calvin cycle and obviously also a key component in cyanobacterial intermediary metabolism. The increase in phosphorylation upon addition of RUBP suggests that

Figure 5.11 : In vitro phosphorylation in *Synechocystis* PCC6803;  
Effect of intermediary metabolites

Autoradiograph showing effects of intermediary metabolites on the *in vitro* phosphorylation pattern seen in high CO<sub>2</sub>-grown cell free extracts of *Synechocystis* PCC6803. Track a, ferricyanide; track b, dithionate; track c, glucose; track d, NaHCO<sub>3</sub>; track e, OMG; track f, ATP; track g, RUBP, track h, NADPH; track i, NADH; track j, NaCl (0.5M), track k, H<sub>2</sub>O.



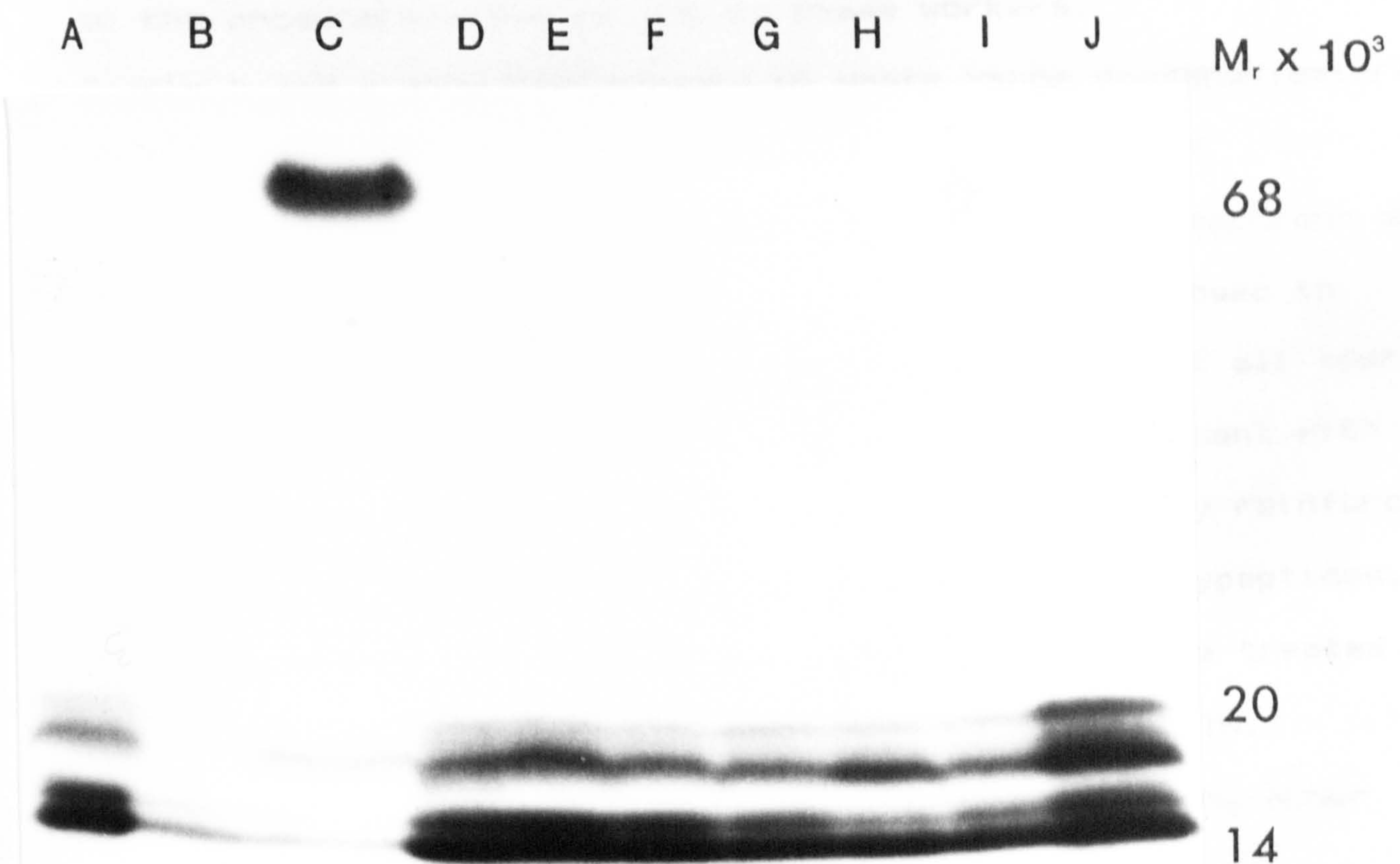
it's level in the cell turns on/off various metabolic pathways. It has previously been shown that RUBP specifically inhibited glucose-6-phosphate dehydrogenase (Pelroy and Bassham, 1972; Pelroy et al., 1976a), a key enzyme interlinking anabolic and catabolic pathways (see Fig. 1.1), although in partially purified preparations of the enzyme RUBP had no effect (Grossman and McGowan, 1975; Schaeffer and Stanier, 1978). The requirement for  $\text{Na}^+$  in the  $\text{Ci}$  concentrating mechanism of various cyanobacteria has been discussed in section 1.5.3, however the role envisaged for this ion has been as part of an electrogenic pump for the uptake of  $\text{HCO}_3^-$  (see Kaplan, 1985), and as the integrity of the cell membrane had been disrupted, the effects seen on *in vitro* phosphorylation by  $\text{NaCl}$  may simply have been due to the effect that this molecule had on ionic balances in the reaction mix.

The effects of a range of sugars and sugar phosphates on *in vitro* phosphorylation was also investigated (see Fig. 5.12). The gel was run at 10 mA for 24 hours to try and improve resolution of the phosphopolypeptides, and the results show that each phosphopolypeptide is clearly discernable. In comparison to the control (track A) none of the sugar phosphates tested had any stimulatory/inhibitory effects on the four low Mr phosphopolypeptides, and neither did fructose or glucose. Galactose, sucrose, maltose and mannose were also tested for their effects on *in vitro* phosphorylation, and as with the sugars and sugar phosphates shown in Fig. 5.12 they too had no effect on the phosphorylation pattern seen in control samples



Figure 5.12 : In vitro phosphorylation in *Synechocystis* PCC6803  
Nature of modification

Autoradiograph showing *in vitro* phosphorylation pattern of high  $\text{CO}_2$ -grown cell-free extracts of *Synechocystis* PCC6803. Track a,  $\text{H}_2\text{O}$ ; track b,  $\text{H}_2\text{O}$  + snake venom phosphodiesterase (100  $\mu\text{g}/\text{ml}$ ,  $37^\circ\text{C}$  for 2 hrs); track c,  $\text{H}_2\text{O}$  + alkaline phosphatase (200 units,  $37^\circ\text{C}$  for 2 hrs); track d, fructose-6-P; track e, fructose-1,6-diP; track f, glucose-1-P; track g, glucose-6-P; track h, fructose; track i, glucose; track j, RUBP



(data not shown). RUBP again stimulated phosphorylation of the Mr 20,000 polypeptide (track J).

Mann et al. (1991) showed that glucose-6-phosphate exhibited a regulatory effect on a phosphopolypeptide Mr 56,000 (p56) by stimulating a protein phosphatase, whereas ribulose-5-phosphate exerted a regulatory effect on the same protein by inhibiting its protein kinase. Fructose-1,6-diphosphate and fructose-6-phosphate were also shown to have an inhibitory role on the phosphorylation of p56 by these workers.

Tracks b and c show the effects of snake venom phosphodiesterase and alkaline phosphatase respectively on the *in vitro* phosphorylation pattern. Although *in vitro* kinase reactions are a good indicator of protein phosphorylation, as opposed to another form of covalent modification, the fact that all four of the phosphopolypeptides proved susceptible to treatment with these enzymes, together with their hot TCA stability reinforces the fact that these are all ester-linked phosphopolypeptides. There is however one band in the alkaline phosphatase treated track that appears to be resistant to this enzyme. This phosphopolypeptide of Mr 68,000 did not appear in any other track, and is thus probably associated with the alkaline phosphatase treatment, as it was also seen in *in vivo* samples treated with this enzyme.

It has previously been shown that the addition of unlabelled ATP results in inhibition of the *in vitro* kinase activity due to competition between the  $^{32}\text{P}$ -labelled and unlabelled ATP (see Fig 5.11). It was decided to try and establish the concentration of

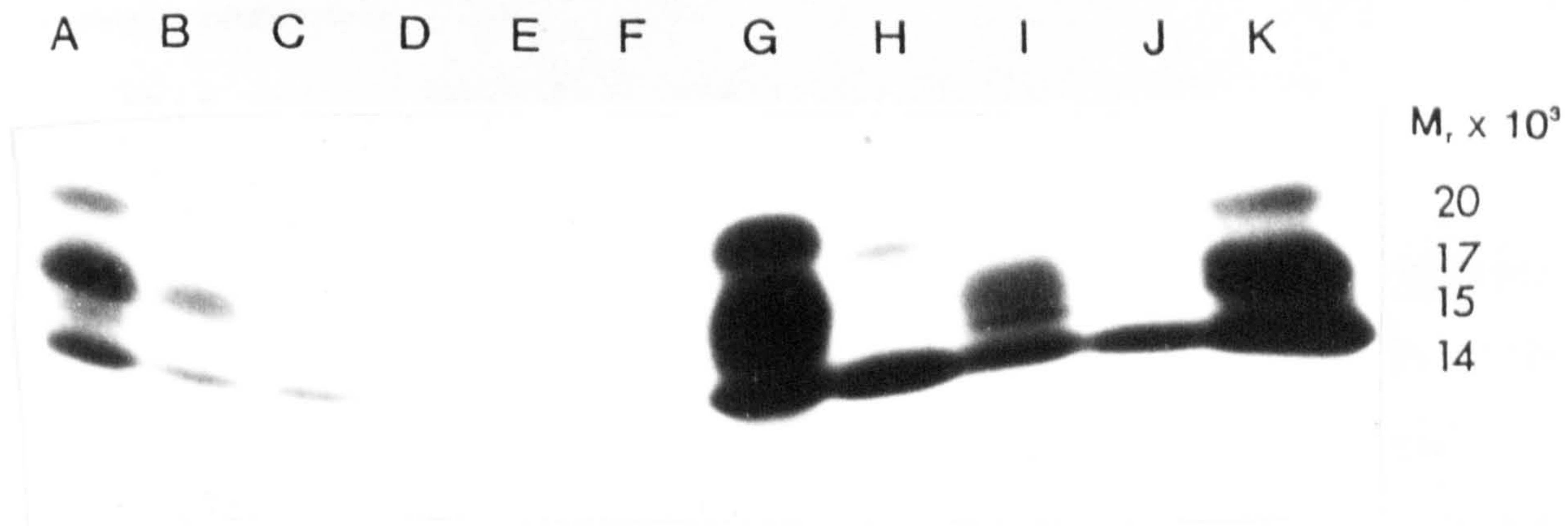


unlabelled ATP that would inhibit the *in vitro* kinase activity. A range of ATP concentrations, from 6.7  $\mu$ M to 67 mM (tracks b-f), Fig. 5.13) were added to the *in vitro* kinase mix. Even very low concentrations of unlabelled ATP inhibited the kinase reaction. Unlabelled ATP at a concentration of 6.7  $\mu$ M led to an inhibition of phosphorylation of the Mr 20,000 and Mr 15,000 polypeptides, and a reduction in the radioisotope incorporation in the Mr 17,000 and 14,000 polypeptides. ATP at a concentration of 67  $\mu$ M led to the inhibition of phosphorylation of all except the Mr 14,000 polypeptide, and at concentrations above this (0.67-67 mM) none of the polypeptides had detectable amounts of radioisotope incorporation. As in previous experiments addition of RUBP stimulated radioisotope incorporation in the four polypeptides (track G), and dithionate inhibited phosphorylation in all but the Mr 14,000 polypeptide (track H). The addition of dithionate and ferricyanide together (track I) resulted in intermediate levels of radioisotope incorporation, similar to the control (track A), and it would seem likely that by manipulating the redox conditions of the *in vitro* kinase mixture that varying levels of radioisotope incorporation could be achieved. Tracks J and K consisted of dithionate and ferricyanide added to a cell free extract of photoheterotrophically grown cells, and the stimulation/inhibition pattern was similar to that seen in Fig. 5.10, however ferricyanide also stimulated phosphorylation of the Mr 20,000 polypeptide, both high CO<sub>2</sub> and heterotrophic extracts had the same response.



Figure 5.13 : In vitro phosphorylation in *Synechocystis* PCC6803;  
Minimum inhibitory concentration of ATP

Autoradiograph showing *in vitro* phosphorylation pattern of high  $\text{CO}_2$ -grown cell-free extracts of *Synechocystis* PCC6803. 6803 Het, cell-free extracts from heterotrophically grown *Synechocystis* PCC6803. Track a,  $\text{H}_2\text{O}$ ; track b, ATP 6.7  $\mu\text{M}$ ; track c ATP, 67 $\mu\text{M}$ ; track d, ATP 0.67 mM; track e, ATP 6.7 mM; track f, ATP 67 mM; track g, RUBP; track h, dithionate; track i, dithionate + ferricyanide; track j, 6803 Het + dithionate; track k, 6803 Het + ferricyanide.

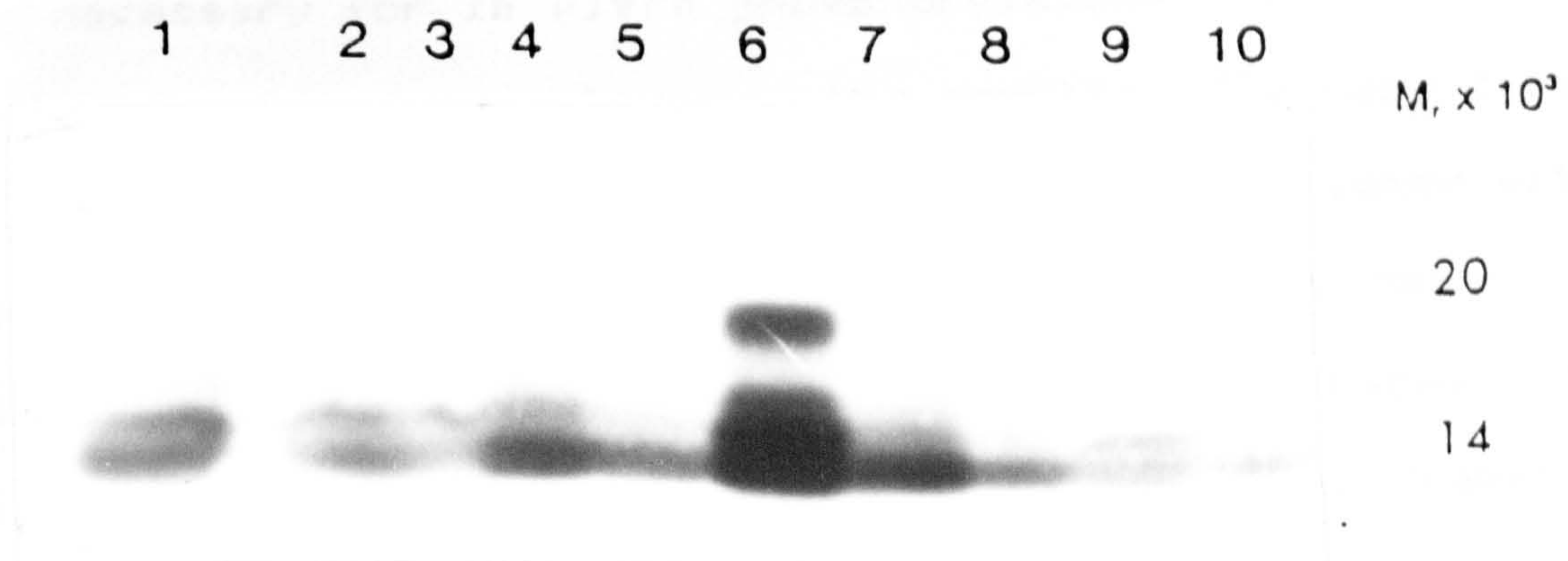


To try and establish whether these low Mr phosphopolypeptides were autophosphorylating or subject to a kinase activity, a kinase dilution experiment was set up. The rationale behind the experiment is that while keeping all other parameters (total protein, buffer concentration and ATP concentration) constant, the protein concentration is reduced over an 8-fold range. If the phosphopolypeptides are autophosphorylating, since total protein and ATP concentration are constant, label incorporation should remain constant, however if a kinase is involved, then dilution of the substrate proteins and kinase should reduce the label incorporation. Tracks 1-4 in figure 5.14 show the result of this experiment. It would appear from the results obtained that the phosphopolypeptide Mr 14,000 is subject to autophosphorylation since the label incorporation remains constant, whilst the label incorporation of the three other low Mr phosphopolypeptides diminishes suggesting a kinase is involved in their phosphorylation. Of the remaining tracks, track 6 had RUBP (12.5 ul) added, which as usual stimulated *in vitro* phosphorylation. Tracks 6 and 7 also had RUBP (10 ul) added, and in addition 10 ul of HPLC fraction 29 (track 6) or HPLC fraction 30 (track 7). To keep the reaction volume to 50 ul, no water was added to these samples. Track 8 contained 12.5 ul of HPLC fraction 29 and track 9 12.5 ul of HPLC fraction 30. These two HPLC fractions probably contain a kinase inhibitor or phosphatase, as they inhibited phosphorylation in HPLC fractions (see Fig. 5.17, section 5.3.3) and it can be seen they negatively modulate the *in vitro* phosphorylation of all four low



Figure 5.14 : In vitro kinase dilution in *Synechocystis* PCC6803

Autoradiograph showing effects of in vitro kinase dilution and HPLC fractions on the pattern of phosphorylation in high CO<sub>2</sub>-grown cell-free extracts of *Synechocystis* PCC6803. Track 1, H<sub>2</sub>O; track 2, 2 x kinase dilution; track 3, 4 x kinase dilution; track 4, 6 x kinase dilution; track 5, 8 x kinase dilution; track 6, RUBP; track 7, RUBP + HPLC fraction 29; track 8, RUBP + HPLC fraction 30; track 9, HPLC fraction 29; track 10, HPLC fraction 30.





Mr phosphopolypeptides, even in the presence of RUBP. A preliminary investigation into the location of these phosphopolypeptides in the cell was undertaken. This involved separating the membrane fraction of the cell free extract from the soluble fraction. This was performed by centrifuging individual *in vitro* kinase reaction mixtures in a Beckman airfuge microcentrifuge (60,000 rpm, 10 min) and separating the pellet, which represented a crude membrane fraction, from the supernatant, the soluble fraction. The centrifugation was performed on samples before and after the *in vitro* kinase reactions to see whether or not components from both fractions were necessary for *in vitro* phosphorylation. Although only a crude separation was achieved, the results were very distinct, with none of the phosphopolypeptides being associated with the soluble fraction (see Fig. 5.15). Tracks 1-5 are control tracks where water was added to the reaction mixture, tracks 6-10 had dithionate added and tracks 11-15 had RUBP added. Tracks 1,6 and 11 were normal *in vitro* kinase reactions. The rest of the tracks contained samples which were either phosphorylated before centrifugation (tracks 2,3,7,8,12 and 13) or in which the soluble and membrane fractions were first separated and *in vitro* kinase experiments performed on the separated fractions (tracks 4,5,9,10,14 and 15). Of these tracks 3,5,8,10,13 and 15 contained membrane fractions, and 2,4,7,9,12 and 14 contained soluble fractions. If the membrane fractions are compared, with the exception of those in which dithionate was added (tracks 6,8 and 10) it would appear that the Mr 14,000 and Mr 17,000



Figure 5.15 : Location of *in vitro* phosphopolypeptides in *Synechocystis* PCC6803

Autoradiograph showing crude location of the phosphopolypeptides from *in vitro* labelled high CO<sub>2</sub>-grown cell-free extracts of *Synechocystis* PCC6803. Tracks 1-5 had water added, in tracks 6-10 the test substance was dithionate and in tracks 11-15 the test substance was RUBP. Tracks 1,6 and 11 contained normal cell-free extract. Tracks 2,4,7,9,12 and 14 contained crude soluble fractions, whilst tracks 3,5,8,10,13 and 15 contained crude membrane fractions. Tracks 2,3,7,8,12 and 13 were *in vitro* kinased before the two fractions were separated, tracks 4,5,9,10,14 and 15 were *in vitro* kinased after separation.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

$M_r \times 10^3$

18  
17  
15  
14





polypeptides in every track have similar radioactive incorporation. However, by comparing the results from the dithionate treated samples, in which radioactive incorporation is reduced, it would appear that radioactive incorporation is greatest in the sample phosphorylated after separation of the soluble and membrane components (track 10). This is supported by the results in track 15, which is the only track in which the Mr 20,000 polypeptide is visible. This indicates that there might be a protein phosphatase associated with the soluble fraction, which regulates, in conjunction with a membrane bound protein kinase, the level of phosphorylation seen in these low Mr polypeptides.

### 5.3.3 Partial purification of phosphopolypeptides

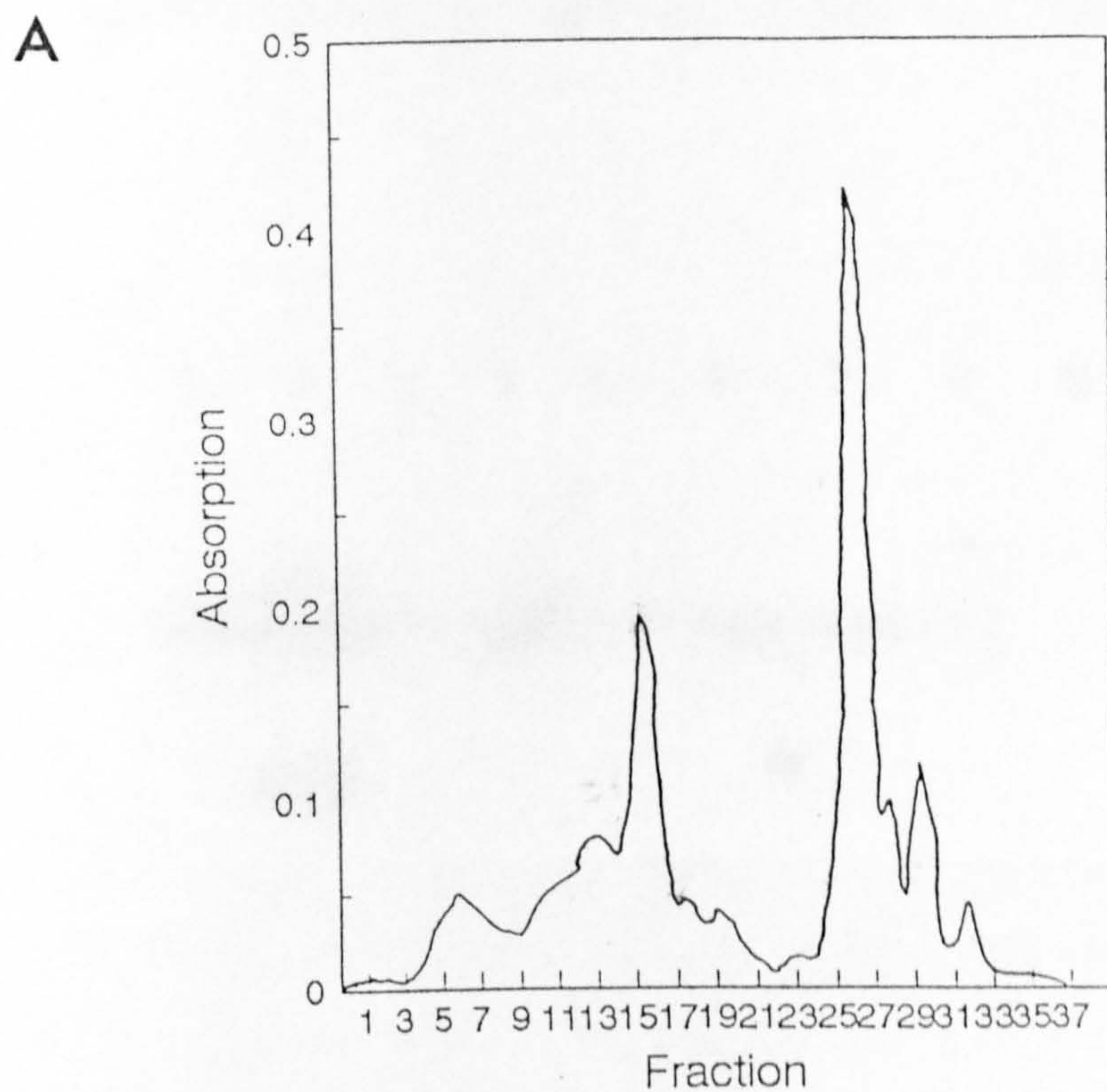
In order to try and further characterize the low molecular weight phosphopolypeptides HPLC, as described in section 5.2.2.1, was performed on soluble extracts prepared from 4 litre batch cultures of high CO<sub>2</sub>-grown *Synechocystis* PCC6803, the HPLC trace represented in Fig. 5.16a. *In vitro* kinase reactions carried out on these fractions identified fractions 17-18 as containing the Mr 14,000 and 17,000 phosphopolypeptides, whilst fraction 16 contained only the Mr 17,000 polypeptide (see Fig. 5.16b). Fraction 22 was the main pigmented fraction (blue), whilst fraction 30, which contained the majority of the protein, contained no phosphopolypeptides.

Figure 5.17 shows the result of mixing fraction 16 with a range of HPLC fractions, in each reaction mix 12.5 ul of each of the



Figure 5.16 : In vitro phosphorylation of HPLC fractions

A) HPLC trace obtained from high CO<sub>2</sub>-grown cell free extract of *Synechocystis* PCC6803. B) Autoradiograph showing *in vitro* phosphorylation of HPLC fractions.



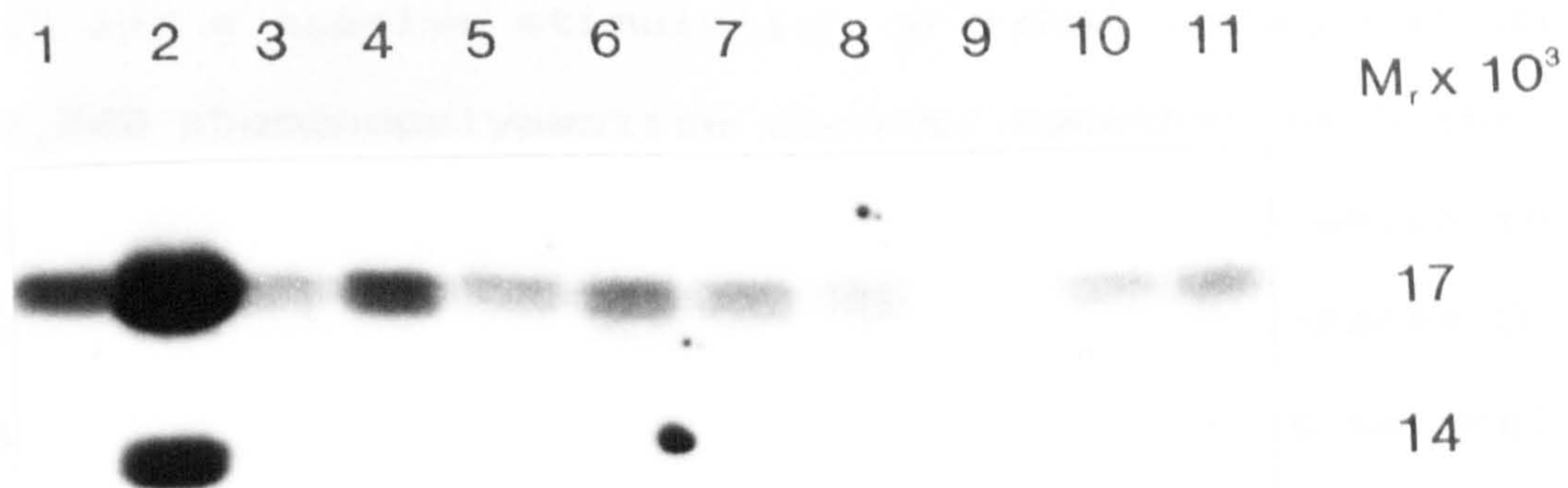
**B**



Figure 5.17 : In vitro phosphorylation of HPLC fractions;  
Identification of a phosphatase/kinase  
inhibitor

Autoradiograph showing the effect of mixing HPLC fraction 16 and other HPLC fractions obtained from a cell-free extract of high CO<sub>2</sub> grown *Synechocystis* 6803.

Track 1, 16; Track 2, 16+18; Track 3, 16+20; Track 4, 16+22; Track 5, 16+24; Track 6, 16+26; Track 7, 16+28; Track 8, 16+30; Track 9, 16+32; Track 10, 16+34; Track 11, 16+36.





two HPLC fractions was present. In tracks 1,2,4 and 6 phosphorylation is stimulated, whilst in tracks 8 and 9, phosphorylation is depressed. Of the tracks where phosphorylation was stimulated, Track 1 contains only fraction 16 (25 ul), and so obviously with twice the amount of fraction 16 there is more label incorporation, and tracks 4 and 6 showed a modest stimulation. Track 2 contains a mixture of fraction 16 and 18 and a massive stimulation of label incorporation into the Mr 17,000 phosphopolypeptide occurs, together with the appearance of the Mr 14,000 phosphopolypeptide, which is a component of fraction 18 (see Fig. 5.16b). In tracks 8 phosphorylation of the Mr 17,000 polypeptide is severely reduced, whilst in track 9, the band corresponding to this polypeptide is completely absent, suggesting that a kinase inhibitor or phosphatase is present. Track 8 contained fraction 30, and track 9 fraction 32, and further work has shown that fractions 29 through to 32 were capable of inhibiting *in vitro* phosphorylation (see also Fig. 5.14). Other experiments in which these fractions were added before and after the *in vitro* kinase reaction suggested that a low Mr phosphatase was present, as similar results were seen on addition before and after the *in vitro* kinase reaction (data not shown).

Further purification of the HPLC fractions by DEAE chromatography, as detailed in section 5.2.2.2 resulted in the majority of the radioactivity being eluted with the kinase buffer, suggesting that most of these low Mr phosphopolypeptides were positively charged. However, on addition of 0.1 M NaCl to



Figure 5.18 : Partial purification of a phosphopeptide from *Synechocystis* PCC6803

Autoradiograph showing the *in vitro* phosphorylation pattern of cell-free extracts of *Synechocystis* PCC6803 eluted from a DEAE affinity chromatography column following a 0.5 M NaCl wash.



$M_r \times 10^3$

17.0

the affinity column, two radiolabelled fractions were eluted, which when dialysed in kinase buffer (3 hrs), run on SDS-PAGE and autoradiographed, produced a band corresponding to the Mr 17,000 phosphopolypeptide (see Fig. 5.18).

#### 5.4 Conclusion

This study has shown in both *Synechocystis* PCC6803 and *Synechococcus* PCC7942 that under growth conditions where the Ci concentrating mechanism is either absent or reduced in its capacity to transport Ci, a number of polypeptides become phosphorylated *in vivo*, making it tempting to speculate that protein phosphorylation may be involved in regulating the process in cyanobacteria, although there is no direct evidence as of yet to support this. In the case of *Synechocystis* PCC6803, these phosphopolypeptides are common to growth conditions where metabolism, although linked by common pathways, is in one case photoautotrophic and in the other photoheterotrophic. 2-deoxy-D-glucose, an analogue of glucose which upon addition to low CO<sub>2</sub>-grown cells caused a decay in the Ci uptake mechanism, had no effect upon the phosphorylation pattern seen, suggesting that phosphorylation of glucose, producing glucose-6-phosphate (G-6-P), and the subsequent build-up of this compound intracellularly, is responsible for the decay of the Ci-uptake mechanism, whilst further metabolism of G-6-P leads to the changes seen in the phosphorylation pattern. Transfer of a

bicarbonate-limited culture into the dark was found to lead to similar, but not identical, changes in the pattern of phosphorylation (N.Silman per. comm.). Such a transfer would lead to a switch from the reductive to the oxidative pentose phosphate pathway, where there would again be no need for an active Ci concentrating mechanism. The common link in all three different growth conditions is the level of operation of the Ci concentrating mechanism.

*In vitro* kinase experiments on cell-free extracts of *Synechocystis* PCC6803 resulted in a number of polypeptides becoming phosphorylated, some of which corresponded in mobility on SDS-PAGE to species phosphorylated *in vivo*, including two low Mr phosphopolypeptides associated with the membrane fraction of the cell. Recently, Mann et al. (1991) found several of the phosphopolypeptides detected during *in vitro* labelling of *Anabaena* PCC7120 corresponded in mobility on SDS-PAGE to species phosphorylated *in vivo*. These authors used a different buffer system (50 mM HEPES NaOH containing 20 mM  $MgCl_2$ ) and recent work in these laboratories using *Synechocystis* PCC6803 has also resulted in similar *in vitro* and *in vivo* labelling patterns (N. Silman per. Comm.)

This *in vitro* phosphorylation could be regulated by altering the redox potential of the reaction mixture, and also by addition of RUBP, which stimulated phosphorylation.

The most important aspect of the work left to complete is obviously the identification of the various phosphopolypeptides. Crude separation of cell homogenates into membrane and soluble



fractions has revealed that spatially, most of the phosphopolypeptides are associated with the membrane fraction of the cell. Non-denaturing PAGE has revealed that a number of native proteins are phosphorylated (data not shown), and the phosphopolypeptides seen following SDS-PAGE are probably components of these native proteins, however attempts to run these phosphorylated native proteins on SDS-PAGE to prove this point were unsuccessful, probably because insufficient protein was loaded onto the non-denaturing gel. HPLC carried out on cell-free extracts resulted in the ability to separate the Mr 17,000 phosphopolypeptide from the other low Mr phosphopolypeptides, and also to the identification of a low Mr phosphatase. DEAE column chromatography identified the Mr 17,000 phosphopolypeptide as being negatively charged, and enabled a much purer preparation of this protein to be obtained. Future work can obviously build on this, leading to the identification of these low Mr phosphopolypeptides and also attempt to identify the higher Mr phosphopolypeptides, which may allow the true role of these phosphorylations to be established.

## Chapter 6

A molecular approach to the study of inorganic  
carbon transport in cyanobacteria

## 6.1 Introduction

### 6.1.1 Mutagenesis

The early suggestion that the difficulty encountered in isolating auxotrophic mutants of cyanobacteria was because the organisms were particularly resistant to mutagens, was unfounded since it became evident that the effectiveness of various mutagens was strain-dependent (see Herdman, 1982).

Stable mutants of cyanobacteria have been obtained by various mutation induction techniques, and appear to affect cyanobacterial DNA in exactly the same way as in other organisms. Chemical mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethyl methane sulphonate (EMS) act specifically at the DNA replication fork, whilst ultraviolet irradiation induces the formation of thymine dimers.

In the past mutants resistant to antibiotics and toxic compounds, temperature sensitive mutants, auxotrophic mutants, mutants deficient in photosynthetic pigments or activity and mutants altered in cellular differentiation have all been obtained, and have proved very useful in the study of specific cellular phenomena (for review see Herdman 1982).

### 6.1.2 Mutagenesis: It's application to inorganic carbon transport

A major difficulty to photosynthetic mutational studies, the fact that most cyanobacteria are obligate autotrophs, is not a problem when studying inorganic carbon transport, as conditional



lethal mutants capable of growth on high  $\text{CO}_2$  but not air levels of  $\text{CO}_2$  can be obtained.

Early work on the unicellular green alga *C. reinhardtii* used 5-fluorodeoxyuridine treatment followed by EMS mutagenesis. Two types of mutant were obtained in this organism (Spalding et al., 1983), both required elevated  $\text{CO}_2$  concentrations for photoautotrophic growth. One was deficient in carbonic anhydrase (CA), designated the *ca-1* gene locus (Spalding et al 1983a), whilst the other had reduced inorganic carbon transport with a correspondingly reduced internal  $\text{CO}_2$  concentration, designated the *pmp-1* gene locus (Spalding et al 1983b). Spalding et al (1985) further characterised these mutants and concluded that internal carbonic anhydrase is an essential component of the  $\text{CO}_2$  concentrating pathway of *C. reinhardtii*. Suzuki and Spalding (1989) have further characterised the mutants *ca-1* and *pmp-1*. They found that although other components of the  $\text{Ci}$ -concentrating system were induced, a mutation in any of the loci concerned with  $\text{Ci}$  transport was sufficient to prevent an increase in the photosynthetic affinity for  $\text{CO}_2$ . They concluded that both loci exhibit at least partially constitutive expression, and that all components of the  $\text{CO}_2$ -concentrating system may be required to significantly affect the photosynthetic affinity for inorganic carbon. A mutant designated CIA-5, isolated by Moroney et al (1989) failed to induce the  $\text{CO}_2$ -concentrating system and was incapable of adaptation to low  $\text{CO}_2$  conditions.

Amongst the cyanobacteria, high  $\text{CO}_2$ -requiring mutants have been

isolated from *Synechococcus* PCC7942 and *Synechocystis* PCC6803, both of which are transformable strains. Mutants have been isolated by the process of chemical mutagenesis (EMS or NTG), followed by penicillin enrichment, formation of colonies by growth on solid media under high CO<sub>2</sub> conditions, and eventual replica plating of surviving cells onto plates incubated in air.

Using *Synechococcus* PCC7942 Marcus et al (1986) obtained the first mutants in inorganic carbon transport, and one of these, E<sub>1</sub>, has been biochemically and physiologically characterised (Marcus et al 1986, Omata et al 1987). This mutant had a high Ci transport activity, and was capable of WT adaptation to low CO<sub>2</sub> conditions but was unable to utilise the intracellular Ci pool for photosynthesis. Ogawa et al (1987) also isolated a mutant of *Synechococcus* PCC7942 that was incapable of utilising the intracellular Ci pool for photosynthesis, however this mutant, RK1, was incapable of adaptive transformation to low CO<sub>2</sub> conditions.

Although the exact nature of the defects is unclear these mutants have been useful in further elucidating the Ci concentrating mechanism, as they enable Ci uptake to be studied whilst CO<sub>2</sub> fixation is blocked (Ogawa and Kaplan 1987). One, mutant O<sub>221</sub>, has also served to demonstrate that a 42kD cytoplasmic membrane polypeptide, induced under low CO<sub>2</sub> conditions, has no obvious role in Ci transport, since although it does not accumulate the 42 kD protein, it is capable of accumulating Ci almost as efficiently as WT cells (Schwarz et al

1988). The first mutants of *Synechococcus* PCC7942 thought to be deficient in the ability to transport Ci were isolated by Abe et al (1988). They selected cells that were high CO<sub>2</sub>-requiring at 40°C but not at 30°C. At 40°C these mutants could not accumulate Ci internally from low external concentrations of Ci (55 µM), whereas the wild-type (WT) cells could. Very recently, one of these temperature-sensitive mutants was transformed to a wild-type phenotype with a clone containing a 36 kb genomic DNA fragment (located 20kb downstream from the structural genes for the subunits of RuBisCO) or a plasmid containing a 3.8kb *Bam*HI fragment from the wild-type strain (Suzuki et al., 1991). These workers also reported that the primary defect in the mutant was in the transport of this intracellular Ci to the site of photosynthetic carboxylation, rather than in accumulation, as reported by Abe et al. (1988). Using *Synechococcus* PCC7942, Price and Badger (1989b) have isolated two phenotypes that accumulate Ci but are apparently unable to generate CO<sub>2</sub> within the carboxysome, having previously reported on a central role for the carboxysome in the CO<sub>2</sub>-concentrating mechanism in *Synechococcus* PCC7942 (Price and Badger 1989a). The high CO<sub>2</sub>-requiring mutants E<sub>1</sub> and O<sub>221</sub> appear to possess defective carboxysomes (Friedberg et al., 1989; Kaplan, 1990), the mutation in O<sub>221</sub> apparently occurring in *rbcL*, the structural gene encoding the large subunit of RuBisCO and its 5'-flanking region (Kaplan, 1990).

Two types of mutant have also been isolated in *Synechocystis* PCC6803 (Ogawa 1990). Type A mutants did not show any CO<sub>2</sub>



uptake, whilst type B mutants were able to transport Ci into the cell but were unable to utilise the intracellular Ci pool. One of the type A mutants, Rkb, has been transformed with a clone (HP-1) isolated from a WT genomic library, resulting in a WT phenotype being produced (Ogawa, 1991a). Complementation tests with subclones derived from HP-1 have enabled the mutation in Rkb to be located within 141 base-pair nucleotides, which from sequencing revealed an open reading frame (ORF) encoding a hydrophobic protein of 80 amino acids (Ogawa, 1991a). A defined mutant (M9) constructed by insertion of a  $Km^r$  cassette into this putative Ci transport gene (designated *ictA*) was unable to transport either  $CO_2$  or  $HCO_3^-$  into the intracellular Ci pool, and biochemical and physiological characterisation of M9 indicate that *ictA* is essential to the transport of both of these Ci species (Ogawa, 1991a). The clone which complements another of these type A mutants, Rka, has also been isolated from the WT *Synechocystis* genome (Ogawa, 1991b). Sequencing of the DNA fragment in the region of the mutation has revealed an ORF (designated *ndh B*) which showed extensive amino acid sequence homology to the *ndh B* genes of chloroplasts and mitochondria [presumed to encode the subunit 2 of NADH dehydrogenase], and physiological characterization of a mutant of the *ndhB* gene has suggested that the gene encodes a protein involved in energization of Ci transport (Ogawa, 1991b). With the exception of Price and Badger (1989b) these workers have used NTG mutagenesis to obtain their mutants. This risks inducing multiple mutations at closely linked sites, and hence

confusing the nature of the mutation. However this is offset by the ease of producing mutants using this chemical. In this study, as well as using NTG, 1,2,7,8-diepoxyoctane (DEO) was also tried. This gives very stable deletion mutants in *Mycobacterium* sp.239 (L.de Boer et al., 1988) and *Rhodococcus* (W.Ashraf, per. comm.).

### 6.1.3 Molecular genetics

The rapid advance in bacterial molecular genetics had led to the development of many new techniques for the study of biological problems, amongst them the gene fusion technology to study the regulation of gene expression (for review see Silhavy and Beckwith, 1985). In this approach an assayable gene product, such as  $\beta$ -galactosidase or antibiotic resistance such as neomycin phosphotransferase is put under the control of the promoter of another gene of interest, and in this way used as a signal to follow expression of the latter gene.

The expression of a given gene in a heterologous host implies that, a) problems arising from any endogenous restriction enzymes have been overcome, b) the gene has a promoter region which is recognised by the host transcriptional machinery, c) the transcript carries, upstream from the translation initiation site, a sequence able to bind host ribosomes and d) the organism from which the gene originated, and the host, must have compatible genetic codes and codon usage.

The presence of restriction endonucleases in cyanobacteria has been extensively established. Wolk et al. (1984) in *Anabaena*

PCC7120 overcame the problems of the restriction endonucleases *Ava*I and *Ava*II by selective removal of their recognition sites before introduction of the vector DNA. *Synechococcus* R2 has a sequence specific endonuclease, *Ani* I (Gallacher and Burke, 1985a). This enzyme is sensitive to *dam* methylation, and by using *dam*<sup>+</sup> strains of *E.coli* for the construction of shuttle vectors this problem was avoided.

Only a few cyanobacterial promoter regions have been published. The sequences in the -35 region seem unrelated to each other and that assigned to the *E.coli* consensus sequence, however the -10 regions resemble each other and the putative RNA polymerase-binding sequence (TATAAT) (see Tandeau de Marsac and Houmard, 1987).

The step by step assembly of initiation complexes has not yet been studied in cyanobacteria, nor have the protein and cofactor requirements been defined. However of the cyanobacterial 16S rRNA sequenced to date, the first published, that of *Synechococcus* PCC6301, has a 3'-end perfectly conserved with respect to *E.coli* 16S rRNA (Tomiooka and Sugaira, 1983)

All cyanobacteria so far studied, appear to use the same genetic code common to both prokaryotes and the eukaryotic chloroplast, for translating nucleotide sequences into polypeptide chains, and the gene fusion technology has been used successfully for the expression of heterologous genes in cyanobacteria.



#### 6.1.4 Construction of cloning vehicles

The first attempt to use cyanobacterial plasmids for the construction of cloning vehicles used the gene encoding ampicillin resistance carried by transposon Tn901 on an *E.coli* plasmid introduced into *Synechococcus* R2 (van den Hondel et al., 1980). The resultant recombinant plasmids obtained (pCH1-pCH5) in the transformants expressed ampicillin resistance, and were the first cyanobacterial plasmids bearing a selectable marker. These plasmids could however only replicate in cyanobacteria, and although *E.coli* plasmids can be transferred into unicellular cyanobacteria, no *E.coli* vector or broad host range plasmid has yet been found capable of autonomous replication in them (van den Hondel et al., 1979; Kuhlemeier et al., 1981; Lightfoot et al., 1987). Daniell et al (1986) reported the transformation of the *E.coli* vector pBR322 into *Synechococcus* PCC6301, however Lightfoot et al. (1987) have been unable to isolate intact molecules of this plasmid in the same strain. Since *E.coli* has figured greatly in recombinant DNA technology, one strategy has been to develop shuttle vectors capable of replication in both *E.coli* and the cyanobacterium under study have been developed, containing a cyanobacterial replication origin whilst another strategy has been to create integrative vectors containing a piece of cyanobacterial chromosomal DNA. The first hybrid vectors (pUC104 and pUC105) were constructed by Kuhlemeier et al (1981) by *in vitro* recombination of *E.coli* vector pACY184 and the cyanobacterial plasmid pUC1. They transform *E.coli* and *Synechococcus* PCC7942 with high frequency.

### 6.1.5 Expression of heterologous genes in cyanobacteria

As well as the examples cited in 6.1.4, Dzelzkalns et al (1984), showed that the expression of the chloramphenicol acetyl transferase (*cat*) gene can be driven in *Synechococcus* PCC6301 by a chloroplast *ps28* gene promoter (also designated *psbA*), consistent with a prokaryotic (cyanobacterial) origin for chloroplast genes. In addition Friedberg and Seijffers (1986), again using the *cat* gene, this time linked to the bacteriophage lambda gene *cI857* showed in *Synechococcus* R2-SPc that expression could be regulated by temperature shift and hence that the lambda promoter was functioning in this cyanobacterium. Gruber et al. (1990) subcloned the manganese superoxide dismutase gene from *E.coli* into an *E.coli*-*Anacystis nidulans* shuttle vector. *A.nidulans* transformants had detectable levels of manganese (Mn) superoxide dismutase and showed resistance to paraquat-mediated inhibition of growth and photobleaching of pigments (paraquat promotes formation of the superoxide radical), however wild-type *A.nidulans* has no detectable Mn superoxide dismutase. Elanskya et al. (1985) and Gallacher and Burke (1985b) have demonstrated the expression of genes originating from Gram-positive bacteria in *Synechococcus* R2. The expression of the *lux* genes from *Vibrio harveyi* and *Vibrio fischeri* into *Anabaena* spp. has been used for the construction of promoter probes for analysis of heterocyst differentiation (Schmetterer et al., 1986).

The most widely used gene fusions involve the *lac* operon (B-galactosidase). Expression of B-galactosidase has been reported in the marine cyanobacterium *Synechococcus* sp. PCC7002 (Buzby et al., 1985), where the *E.coli lacZ* gene was expressed on a plasmid vector, and has been used to assess the effect of light intensity and nitrogen availability on *cpc-lacZ* gene fusions in the same organism (Gasparich et al., 1987). Scanlan (1988) has reported expression of B-galactosidase from both the *Synechococcus* R2 chromosome and a multicopy plasmid. Such studies have shown that *lac Z* can be used to monitor gene expression in cyanobacteria and these *lac Z* gene fusion vectors are the vectors we have set out here to modify and develop to identify presumptive CO<sub>2</sub>-regulated promoters.

#### 6.1.6 The development and use of lac fusions in the study of biological problems

There are several reasons why the *lac* genes have been widely used for gene fusion studies. The *lac* operon is one of the most intensively studied genetic systems, and hence many genetic and biochemical aspects of the system are known. Several indicator media available for detecting sugar metabolism can be utilised because lactose, the substrate of the system, can be used by *E.coli* as the sole carbon source. However, the most important feature of the lactose system is the ability to generate numerous functional analogues of lactose in which the glucose moiety is substituted. The analogue o-nitrophenyl-β-D-galactopyranoside (ONPG) is a substrate of



B-galactosidase, which upon hydrolysis by the enzyme yields o-nitrophenol, a yellow product, the resultant enzyme assay system being one of the most sensitive available.

In developing a method for constructing *lac* fusions, two problems must be solved. The *lac* genes must first be moved to a chromosomal site that is closely linked to the target gene, and they must be in the same transcriptional orientation, and secondly a genetic arrangement that creates the appropriate novel fusion joint must be selected. The discovery of transposable genetic elements in the early 1970's provided a method of moving the *lac* genes to any position on the chromosome. Using this technique Casadaban and Chou (1984), constructed a defective phage, Mu d2(Ap *lac*), which when inserted into a target gene in the correct orientation generated a hybrid protein in a single step, providing the reading frame was correct. These *in vivo lac* fusions using transposable phage are however unstable, since secondary insertions occur, complicating genetic analysis. Since then Youngman et al. (1985) in *Bacillus subtilis* and Kroos and Kaiser (1984) in *Myxococcus xanthus* have extended the use of transposons to create *in vivo lac* fusions in other organisms, however the lack of an efficient transposable element for use in cyanobacteria, has meant that these *in vivo* studies are not, as yet possible in these organisms.

The advent of recombinant DNA technology has, however, provided a powerful methodology for the construction of novel DNA joints *in vitro*. The aim of these constructions, as with the *in vivo*

ones, was to devise a procedure in which the creation of a desired novel joint caused activation of a particular enzymic activity. With *lacZ* this required the physical separation of the *lacZ* sequences required to specify a functional enzyme from the signals that direct transcription and translation initiation. The standard vector for constructing *lacZ* fusions *in vitro* contains a *lacZ* gene truncated at the 5' end at a site before codon 26 (which separates the essential *lacZ* coding sequence from the expression signals). Consequently the gene is missing a promoter, a ribosome-binding site and an ATG initiation codon. At the point of the truncation, synthetic DNA sequences containing multiple restriction enzyme cleavage sites are inserted (see Fig. 6.1). Thus, if a 5' coding sequence containing the required expression signal is cloned into one of the restriction enzyme cleavage sites, so that transcription and translation facilities are restored across *lacZ*, a hybrid protein is produced, with B-galactosidase activity. The "strength" of these promoter sequences carried on the inserted DNA determines, along with the strain specificity involved, the level of B-galactosidase activity, provided it is free from influences that are not promoter-specific, such as those relating to the stability and translation of mRNA. As well as the vectors described above, which have been used to create gene fusions specifying hybrid proteins, vectors for the creation of operon fusions have also been created (see Casadaban et al., 1983). These are less popular because it is often difficult to distinguish strains carrying the desired fusion

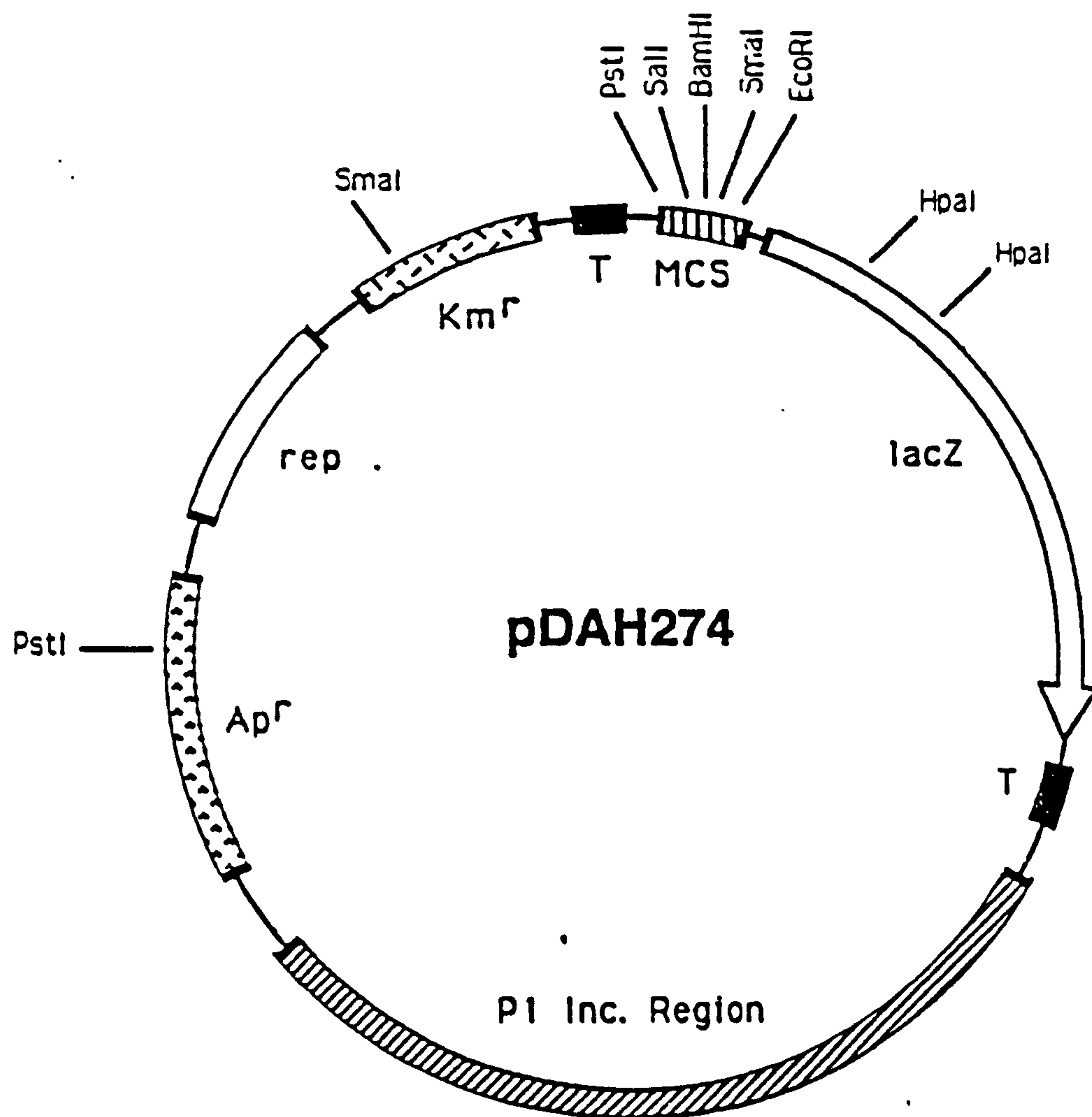


Figure 6.1 Restriction map of pDAH274 showing multiple restriction cleavage sites



from parent strains, since background expression of an intact *lacZ* gene, even in the absence of a recognisable promoter is quite high when carried on a multicopy plasmid.

*lac* fusions have been used for a wide variety of purposes. These include the study of the regulation of another gene or operon, to detect genes which are subject to a particular regulatory signal, to study the localisation of a protein to an intracellular compartment or the extracellular space and to detect a gene, the protein product of which is known, but for which there is no easy method for localising or cloning the gene (for review see Silhavy and Beckwith, 1985)

#### 6.1.7 Physiological regulation of gene expression

In regulatory systems such as the *lac* operon there is a direct chemical relationship between regulatory effector molecules, which are substrates or end products (or metabolic derivatives) and the regulated genetic system. In more complex systems, the regulatory effector is not a metabolite of the regulated system, but acts as a "symbol" of the cells nutritional status, such as cyclic AMP acting as a symbol of glucose deficiency in *E.coli* (Pastan and Adhaya, 1976).

Cyanobacteria very often do not alter enzyme levels in response to changes in their environment, which if inflicted upon *E.coli* would dramatically affect enzyme formation and hence gene expression. Carr (1973) interpreted this behaviour as a cause of their obligately photoautotrophic tendencies, whereas Doolittle (1979) thought this failure to regulate gene expression in

certain pathways, a consequence rather than a cause of autotrophy. However, certain examples of regulated gene expression are well documented in cyanobacteria. Light intensity and colour quality have long been known to affect gene expression in cyanobacteria. Myers and Kratz (1955) showed phycocyanin (PC) levels varied over a 4-fold range in response to variation in white light intensity, and chromatic adaptation, where light quality alters phycocyanin:phycoerythrin (PE) ratios to maximise light harvesting ability has also been well documented (see Bogorad, 1975; Tandeau de Marsac et al., 1988)). Biochemical experiments have shown that the induction of proteolytic enzymes can occur in response to specific physiological conditions. It has been shown in *Anabaena* sp. that nitrogen starvation induces a specific proteolytic degradation of phycobilisomes and/or cyanophycin (Wood and Haselkorn, 1980; Gupta and Carr, 1981).

Gene fusions can allow a more detailed analysis of the environmental factors regulating a gene than is possible with biochemical methods, in which the assay of the gene product under study can be laborious or in some cases non existent. The effect of light intensity and nitrogen availability on *cpc-lacZ* gene fusions in *Synechococcus* sp. PCC7002 (Gasparich et al., 1987) has already been mentioned (see 7.1.5), and a light induced mechanism is responsible for the expression of the genes responsible for carotogenesis in *Myxococcus xanthus* (Balsalobre et al., 1987)

Kuritzkes et al., 1984, used *glp-lac* fusions to study anaerobic control of the *glp* gene in *E.coli*, whilst in *E.coli* K12, phosphate starvation inducible genes have been identified by analysis of *psi::lacZ* transcriptional fusions (Metcalf et al., 1990). Scanlan (1988) has shown, using pieces of *Synechococcus* chromosomal DNA fused to the promoter probe pLACPB1, differential expression of *lacZ* in response to iron and magnesium limitation,

A number of authors have reported on genes regulated by oxygen levels. Hudig et al. (1987) showed, in *Rhodobacter capsulatus*, that the formation of cytochrome c oxidase is regulated by oxygen tension and light intensity, whilst the regulation of cytochrome activity is correlated to oxygen tension only. In *E.coli* superoxide dismutase (*soi*) gene fusions with *lacZ* were constructed which were inducible by oxidative stress (Kogama et al., 1988). In the same organism, the gene encoding the *Vitreoscilla* hemoglobin-like molecule (VHb) was cloned and functionally expressed. Using direct analysis of mRNA levels and *lacZ* gene fusions, it was found the promoter was maximally expressed under microaerobic conditions (Khosala and Bailey, 1989).

In *C.reinhardtii* cDNA clones for the periplasmic carbonic anhydrase were found to be regulated by environmental CO<sub>2</sub> concentrations as well as light at the level of mRNA abundance (Fukuzawa et al., 1990).

Bhriain et al (1989) have shown, using *E.coli*, an overlap between the response to osmotic shock and to anaerobic stress.



They suggest there is a class of "stress regulated" genes that are regulated by a common mechanism in response to different environmental conditions, and furthermore that this regulatory overlap is mediated by changes in DNA supercoiling in response to these environmental stresses

#### 6.1.8 Promoter-probe vectors

The DNA sequences of promoters contain signals that are recognised by RNA polymerase, as well as containing information that determines their efficiency (Rosenberg and Court, 1979). The efficiency of a promoter can be modulated in many ways (see de Boer and Shephard, 1983). Systems developed to assay this modulation have been described for various bacteria and yeast e.g. in *E.coli* using the galactokinase gene (*galK*) (de Boer, 1984), chloramphenicol and tetracycline resistance (Brosius, 1984), and B-galactosidase, the most commonly used assay (Linn and Pierre, 1990); in *Caulobacter crescentus* using neomycin phosphotransferase II (Bellafato et al., 1984); in *Zymomonas mobilis* using B-galactosidase (Conway et al., 1987); in *Myxococcus xanthus* using B-galactosidase (Kroos and Kaiser, 1984); in the eubacteria *Deinococcus radiodurans* using B-galactosidase (Lennon and Minton, 1990); in the yeast *Saccharomyces cerevisiae* using B-galactosidase (Myers et al., 1986) and *Bacillus* spp. using luciferase (Carmi et al., 1987). Promoter probes have also been used for the analysis of divergently arranged promoters, which direct transcription bidirectionally from closely spaced sites, and in this case the

$\beta$ -galactosidase gene was employed in combination with either the galactokinase gene or alkaline phosphatase gene (*phoA*) (Schneider and Beck, 1986). Amongst the cyanobacteria, the luciferases from *V.harveyi* and *V.fischeri* have been used to assay expression in *Anabaena* sp. of various promoters (Schmetterer et al., 1986 and Elhai and Wolk, 1990). Using this technique they were able to monitor the activity of single genes in single cells of the cyanobacterial filament. Others working with cyanobacteria have used chloramphenicol acetyl transferase (Dzelzkalns et al., 1984; Ferino and Chauvat, 1989; Lang and Haselkorn, 1991)) and  $\beta$ -galactosidase (Gasparich et al., 1987 and Scanlan, 1988).

The induction of a  $\text{CO}_2$  concentrating mechanism in cyanobacteria (see section 1.5) when transferred from high to low  $\text{CO}_2$  (or  $\text{Ci}$ ) growth conditions, must involve regulated gene expression. Since no promoters involved in the  $\text{Ci}$  concentrating mechanism have been identified, the approach of introducing cyanobacterial chromosomal DNA in front of a promoterless *lacZ* was adopted, to identify  $\text{CO}_2$ -regulated promoters by differential *lacZ* expression under high and low  $\text{CO}_2$  conditions.

## 6.2 Materials and Methods

All molecular biological techniques and transformation procedures were performed as described in Chapter 2, except Southern blotting which is described below. Protein Estimation was by the BioRad method (Section 2.14.1)

### 6.2.1 Chemical mutagenesis

#### 6.2.1.1 NTG mutagenesis

*Synechococcus* R2 and *Synechocystis* PCC6803 were grown to logarithmic phase in BG-11 under high CO<sub>2</sub> conditions, and resuspended to a final concentration of  $1-2 \times 10^7$  cells ml<sup>-1</sup>. NTG (0.5 mg and 0.03 mg ml<sup>-1</sup> final concentration) was added to the cultures, which were then reincubated under high CO<sub>2</sub> conditions. Samples (1 ml) were removed at intervals, washed twice with BG-11, and appropriate dilutions spread onto BG-11 plates supplemented with 10 mM NaHCO<sub>3</sub>, incubated in the light in gas bags supplied with 5% CO<sub>2</sub>:95% air, and the number of survivors determined against untreated control cultures. Herdman and Carr (1972) showed in cyanobacteria that maximum mutation frequency was correlated with lethal effect, and the point at which less than 1% of the culture was viable was used for NTG mutagenesis (see Fig. 6.2).

#### 6.2.1.2 Diepoxyoctane mutagenesis

The mutagen diepoxyoctane (DEO) (97% solution v/v), a gift from W. Ashraf, this laboratory, was added to an exponentially growing culture of *Synechococcus* R2, at a final concentration of



0.1 or 0.2 % (v/v).

A killing curve was established as in 6.2.1.1, cells were washed four times as opposed to two with NTG.

#### 6.2.2 Isolation of mutants

NTG treated cells (0.3 mg/ml) were washed twice with BG-11, resuspended in BG-11 supplemented with 10 mM NaHCO<sub>3</sub>, and incubated under 5% CO<sub>2</sub> for two days to allow segregation of any mutant genome, after which the cells were transferred to low CO<sub>2</sub> conditions, left for 16-24 hrs to allow depletion of any endogenous supplies of Ci, and 1 mg ml<sup>-1</sup> ampicillin added. After 2 days, the remaining cells were harvested by centrifugation, washed twice with BG-11, and plated onto BG-11 plates supplemented with 10 mM NaHCO<sub>3</sub>, placed in gas bags supplied with 5% CO<sub>2</sub> (high CO<sub>2</sub> plates), and incubated in the light until colonies appeared. Colonies were replica plated onto BG-11 plates under high or low CO<sub>2</sub> conditions to identify presumptive Ci concentrating mutants.

#### 6.2.3 B-galactosidase assays

Quantitative B-galactosidase activity was assayed routinely using 4-methyl umbelliferyl-B-D-galactoside (MUG) (see Youngman et al., 1985), although o-nitrophenyl-B-D-galactopyranoside (ONPG), as described by Miller (1972), was occasionally used. All assays on solid media (qualitative), were performed using MUG, as the colour of cyanobacterial colonies interferes with the colour reactions produced using X-gal plates.

Colonies appearing after transformation with the promoter probes pLACPB1 and pLACPB2, were restreaked onto BG-11 plates containing 7.5 ug Cm/ml (low CO<sub>2</sub> plates), and then replica-plated onto BG-11 plates containing Cm + 10 mM NaHCO<sub>3</sub> (high CO<sub>2</sub> plates). These were then placed inside sealed gas bags, before gassing with air or 5% CO<sub>2</sub> in air. After the transformant colonies had grown up, they were treated as below.

i) MUG assays

When MUG is hydrolysed by B-galactosidase, a methylumbelliferone is generated which is highly fluorescent under long-wavelength UV light. This allows sensitive visual detection of B-galactosidase activity in bacterial colonies, and in contrast with colour reactions produced by bacterial colonies grown on X-gal, which reflect substrate hydrolysis throughout the development of the colony, MUG assays can detect the quantity of B-galactosidase present at the time the substrate is applied. MUG was applied after corresponding high and low CO<sub>2</sub> transformant colonies had developed (usually five days), by spraying the plates with a 10 mg ml<sup>-1</sup> MUG solution prepared in DMSO. Plates were held 30 cm away from the atomizer nozzle, and a fine spray of MUG projected over the surface of the plate. After 5 minutes, plates were visualised under long wavelength UV light and photographed. This initial screening allowed a preliminary identification of transformants exhibiting CO<sub>2</sub>-regulated expression of B-galactosidase. These "interesting" transformants were then grown in liquid medium under high and

low CO<sub>2</sub> conditions, and B-galactosidase activity assayed throughout the growth curve using a quantitative MUG assay. Quantitative fluorimetric assay for B-galactosidase specific activity was carried out as follows: 1 ml of a bacterial culture of a known optical density was pelleted by centrifugation for 1 min in an Eppendorf centrifuge, and the cells resuspended in AB buffer (60 mM K<sub>2</sub>HPO<sub>4</sub>, 40 mM KH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl). If normal activity was expected, cells were resuspended in 500 ul AB buffer, but for low activity cells were resuspended in 100 ul AB buffer. If high activity was expected, cells were diluted 10-20x in AB buffer after resuspension. 50 ul of the resuspended sample was transferred to an Eppendorf tube, and 10 ul of 0.4 mg ml<sup>-1</sup> MUG in DMSO added to start the reaction. After incubation at room temperature for 100 min, the reaction was "stopped" by removing 50 ul and diluting into 2.45 ml AB buffer. Samples were read in a Perkin-Elmer LS-5 luminescence spectrophotometer (Excitation slit width 5, wavelength 366 nm; Emission slit width 10, wavelength 445 nm). For a negative control 50 ul AB buffer was treated as if it were a sample. The fluorimeter was zeroed against AB buffer as a blank, and then a reference sample of 4-methylumbelliferone (Sigma) at a concentration of 400 nM was placed in the machine. The fluorimeter reading was adjusted to give a reading of 400, which meant the fluorimeter read the actual concentration of hydrolysed substrate in nanomoles. The specific activity was described as one picomole of MUG hydrolysed per ml of culture sample per minute, normalised for culture density.



$$\text{units} = \frac{(\text{fluorimeter display}) (1000) (2.5/1000) (60/50)}{(\text{ml of culture per assay}) (\text{duration of assay/min}) (\text{OD}_{750})}$$

#### ii) ONPG assay

A cell lysate (50  $\mu\text{l}$ ) was dispensed into Eppendorf tubes, and the volume made up to 500  $\mu\text{l}$  with Z buffer (see Table 6.1). 100  $\mu\text{l}$  of a fresh solution of ONPG (4  $\text{mg ml}^{-1}$  in Z buffer) was added to each tube, the tubes incubated at  $37^{\circ}\text{C}$ , and the time taken for a faint yellow colour to appear recorded (up to 20 min). The reaction was stopped by the addition of 250  $\mu\text{l}$  1M  $\text{Na}_2\text{CO}_3$ , the absorbance read at 420 nm, and data expressed as the increase in  $\text{OD}_{420} \text{ min}^{-1} \text{ ml}^{-1}$ . Control tubes contained 100  $\mu\text{l}$  Z-buffer instead of 100  $\mu\text{l}$  ONPG.

Table 6.1     Constituents of Z-buffer (per litre)

$\text{Na}_2\text{HPO}_4$	8.52g
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	6.24g
KCl	0.75g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25g
B-mercaptoethanol	2.7 ml
Do not autoclave	[pH 7.0]

#### 6.2.4 Southern blotting

Chromosomal DNA was isolated from *Synechococcus* R2 (see Section

2.19), and subjected to restriction endonuclease digestion with *Bam*H1, *Xho*I, *Eco*RI and *Eco*RV for 6 hours, 37°C, and *Sau*3A, 20 min at 37°C.

After agarose gel electrophoresis the DNA was partially hydrolysed by acid purination - the gel was soaked in 0.25 M HCl for 20 minutes, by which time the bromophenol blue band had turned yellow. Further denaturation was achieved by three 15 minute washes in 1.5 M NaCl, 0.5 M NaOH, after which the gel was neutralised by three 15 minute washes in a solution of 1 M Tris pH 7.4, 3 M NaCl. All of the above treatments took place at room temperature (see Maniatis et al., 1982). Transfer of DNA from agarose gels to nitrocellulose filters was as described by Southern (1975), SSC (20xSSC contained in g/L<sup>-1</sup>: NaCl 175.3 g, sodium citrate 88.2 g, pH 7.0) was the transfer buffer used.

#### 6.2.5 Radiolabelling DNA fragments

DNA was labelled to high specific activity using an adaptation of the technique described by Feinberg and Vogelstein (1984). It was not necessary to isolate individual pieces of plasmid DNA, since the hybridisation reaction was only performed to make sure cyanobacterial chromosomal inserts were present in the various transformants. To this end, plasmid DNA was isolated from the transformants by the large scale isolation method from *E.coli*, as described in Section 2.20. Plasmid DNA (1.5 ul) was then digested with *Xho*I for 2 hours, as described in 2.17.1. The volume was then made up to 16.25 ul with sterile distilled water and the labelling reaction carried out by the addition of: 5 ul

OLB buffer (Table 6.2), 1 ul BSA, 1 ul Klenow large fragment DNA polymerase I (Amersham International) and 2.5 ul  $^{32}\text{P}$ -dCTP (10 uCi/ul). Incubation was carried out overnight at room temperature. To the probe was added 0.2 ml TE and 0.1 ml salmon sperm DNA (10 mg/ml), the mixture was boiled for 10 min, placed on ice for 10 min, and then added to hybridisation fluid (see 6.2.6)

#### Table 6.2 Composition of OLB buffer

OLB buffer is composed of the following solutions, A:B:C in the ratio 10:25:15.

Solution O: 1.25 M Tris-HCl pH 8.0, 0.125 M  $\text{MgCl}_2$ . Stored at 4°C.

Solution A: 1 ml solution O + 18 ul 2-mercaptoethanol + 5 ul dATP, 5 ul dTTP, 5 ul dGTP (each triphosphate previously dissolved in 3 mM Tris-HCl, 0.2 mM EDTA, at a concentration of 0.1 M, pH 7.0). Stored at -20°C.

Solution B: 2 M Hepes (titrated to pH 6.6 with 4 M NaOH). Stored at 4°C.

Solution C: Pentadeoxyribonucleotides (Pharmacia) 50 OD units in 550 ul TE buffer, giving a concentration of 90 OD units/ml.

#### 6.2.6 DNA-DNA hybridisation analysis

Prehybridisation and hybridisation of filters was carried out as



described by Maniatis *et al.* (1982), except for the prehybridisation and hybridisation solutions which contained: 5 x SSPE (3 M NaCl, 0.2 M  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 20 mM EDTA pH 7.4), 5 x Denhardt's solution (5 g Ficoll 400, 5 g polyvinylpyrrolidone-10, 5 g BSA (Pentax Fraction V) in 500 ml distilled water = 50 x stock solution) and 0.1 % SDS. The prehybridisation incubation period was 4 hours at 65°C. The hybridisation fluid was exactly the same as the prehybridisation fluid except that it also contained  $^{32}\text{P}$ -labelled denatured probe DNA and 100  $\mu\text{l}$  denatured salmon sperm DNA (10 mg/ml). Hybridisation took place at 65°C and incubation was for 16 hr.

For homologous probes, using a high stringency, filters were washed in 0.1 x SSPE, 0.1% SDS for 15 min, and then again for 90 min at 65°C. Filters were exposed to X-ray film (Fuji RX) in an X-Ray cassette with an intensifier screen, and stored at -70°C. The film was developed using the same chemicals and procedures as in Section 2.16.4.

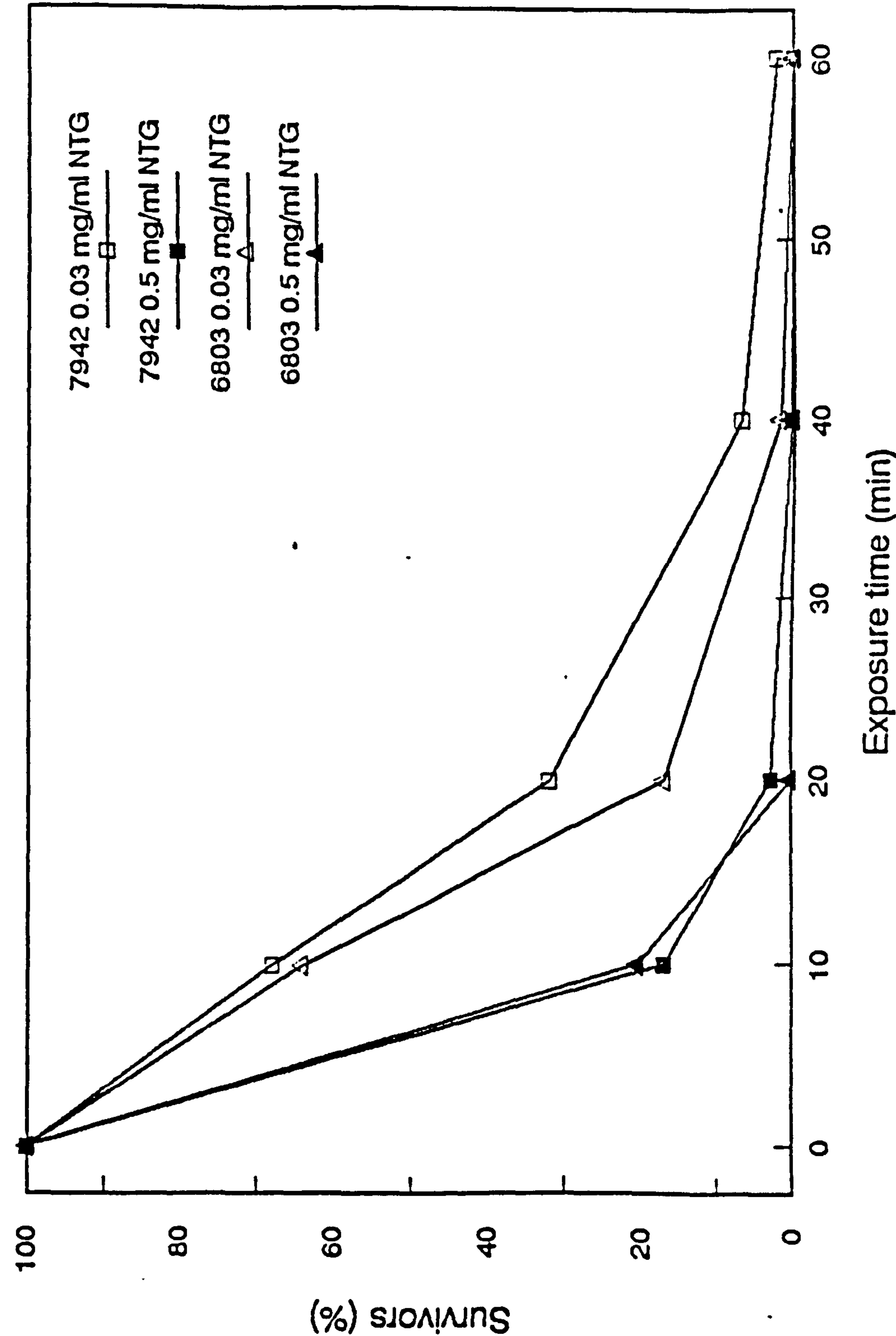
### 6.3 Results and Discussion

#### 6.3.1 Chemical mutagenesis of *Synechococcus* R2 and *Synechocystis* PCC6803

*Synechococcus* R2 and *Synechocystis* PCC6803 were treated with 0.03 and 0.5 mg/ml NTG, and a killing curve established (Fig. 6.2). As expected, the lethal effect of exposure to 0.5 mg/ml NTG was greater than exposure to 0.03 mg/ml NTG, however, even at the lower concentration of NTG used, the viability of *Synechocystis* PCC6803 was down to 1% after 40 minutes incubation, and that of *Synechococcus* R2 by 60 minutes, and so 0.03 mg/ml NTG was the concentration used for further experiments, at an incubation time resulting in 1% viability. DEO (0.1%) gave a 99% kill after a 40 min incubation, whilst at a concentration of 0.2% (v/v) a 99.5% kill was achieved in 20 min. *Synechococcus* R2, therefore seems more sensitive to the effects of DEO than *Mycobacteria*, in which a 95% kill was achieved with a 0.2% solution in 75 min (L.de Boer et al., 1988) or *Rhodococcus* in which a 95% kill was achieved in 20 min with a 0.2% (v/v) solution (W.Ashraf, per.comm.).

After penicillin enrichment (Section 6.2.2), between 1000 and 15,000 colonies of both *Synechococcus* R2 and *Synechocystis* PCC6803 appeared, which represents an 85-99% enrichment of the mutagenised cells, similar to that seen by Herdman and Carr (1972). The colonies that appeared were very slow growing, and were still very small after 5-7 days. Some had a different colony morphology to wild-type (WT) cells and a few were pigment mutants (see Fig 6.3).

Figure 6.2 : Survival of *Synechococcus* PCC7942 and *Synechocystis* PCC6803 to addition of NTG





500 of these presumptive mutants from both organisms were screened for the ability to grow under low CO<sub>2</sub> conditions (including some morphology and pigment mutants). All grew on BG-11 plates, indicating that no mutants impaired in the ability to transport and concentrate CO<sub>2</sub> had been obtained. CO<sub>2</sub>-uptake experiments on five of the slower growing colonies (including a morphology and pigment mutant) proved this to be the case, all having uptake rates similar to WT cells. This inability to obtain mutants defective in their ability to grow under low CO<sub>2</sub> conditions was puzzling, since a number of workers (see Section 6.1.2) have obtained mutants. Either i) no CO<sub>2</sub>-uptake mutants were obtained or ii) the screening procedure was inadequate. However with the advent of the promoter probe studies the probable reason for this became apparent. Plates incubated under low CO<sub>2</sub> conditions in the controlled temperature room were in the same room as those plates incubated under high CO<sub>2</sub> conditions and also the continuous-culture vessels, and hence although not gassed directly with 5% CO<sub>2</sub>, were in an environment which probably had a raised level of CO<sub>2</sub>, enabling any mutants that might have been present to grow on both the high and low CO<sub>2</sub> plates. Since penicillin enrichment allowed a number of different mutant types to grow, the chances of picking a CO<sub>2</sub>-uptake deficient mutant was minimal. Any future work performed in these laboratories will have to ensure that low CO<sub>2</sub> plates are incubated in a separate room, or as was done for the promoter probe studies, in a sealed gas-bag gassed with air to provide a positive pressure inside, preventing influx of any 5%



Figure 6.3 : Morphology and pigment mutants of *Synechococcus* PCC7942 obtained following NTG mutagenesis

A, Morphology mutant; B, Pigment mutant.

A



B

PM  
↓





CO<sub>2</sub> in air.

### 6.3.2 Construction of a *lacZ* promoter probe for use in *Synechococcus* R2-SPc

Scanlan (1988) constructed a number of vectors for use in *Synechococcus* R2-SPc making use of *lacZ* as a reporter gene, including the promoter probe, pLACPB1. However he reported endogenous *lacZ* activity of pLACPB1 in *Synechococcus* R2, and he suggested constructing a vector with transcription termination signals both upstream of *lacZ* and the site of insertion of chromosomal DNA, and downstream of *lacZ*, which might reduce this endogenous *lacZ* activity. pDAH274 (see Fig. 6.1), a gift from D.A.Hodgson, this laboratory, contains such transcription terminators, and its suitability for assessing *lacZ* activity, led it to be used in this study. pDAH274 is incapable of independent replication in cyanobacteria (Table 6.3). To be capable of replication in *Synechococcus* R2-SPc, either chromosomal DNA from *Synechococcus* R2-SPc would have to be inserted into the plasmid to enable integration into the cyanobacterial chromosome, or an independently replicating shuttle vector constructed.

Scanlan (1988), using pDAH216, similar to pDAH274 but lacking transcription terminators, found the transformation frequency using the former technique to be very low, and furthermore no differential *lacZ* activity was observed, in contrast to the promoter probe pLACPB1.

It was therefore decided to construct a promoter probe (Fig.

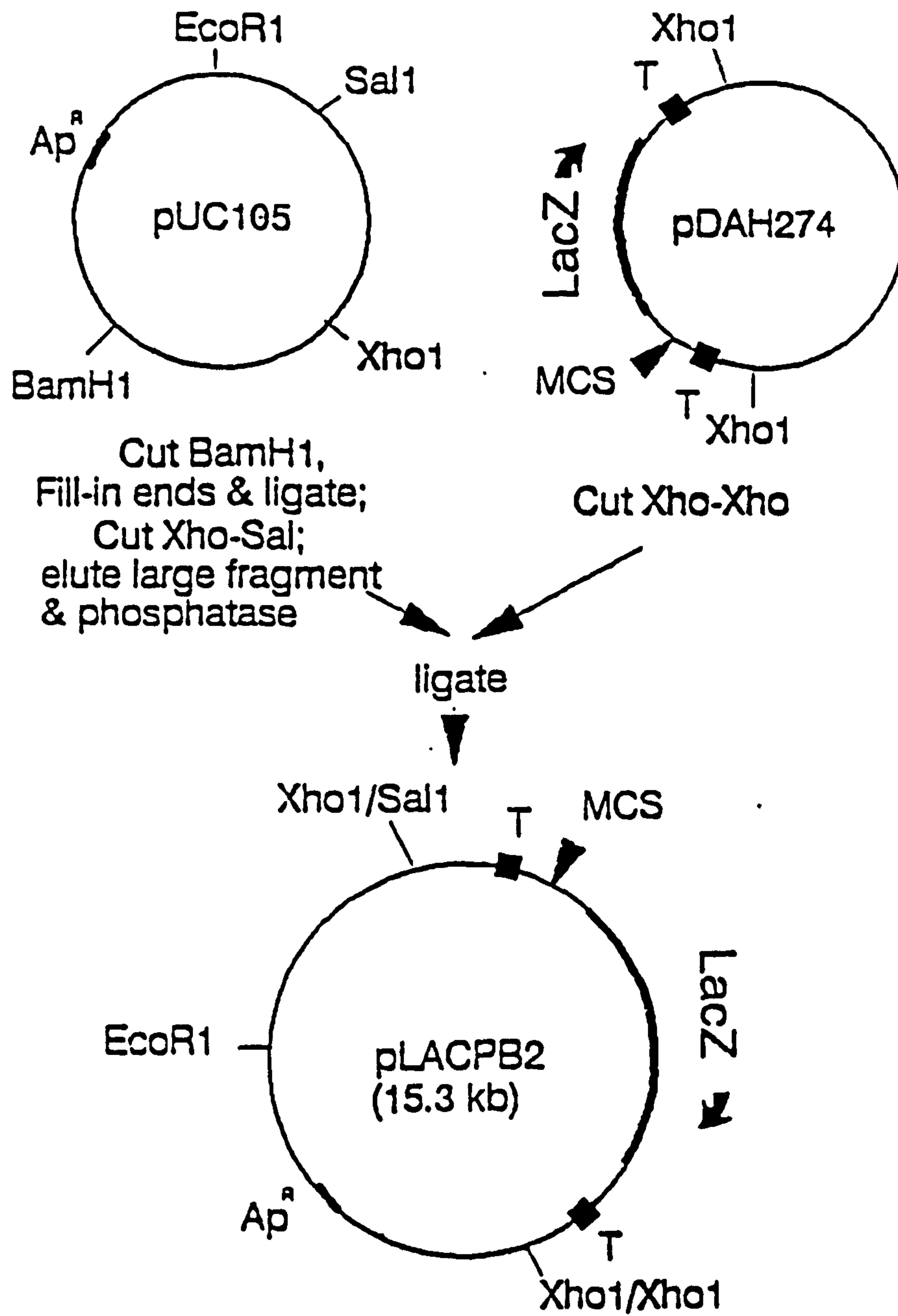


6.4), based on the shuttle vector pUC105 (Kuhlemeier et al., 1981) containing the promoterless *lacZ* gene from pDAH274. The *Bam*HI site in pUC105 was removed by filling in cut ends using the Klenow fragment of *E. coli* DNA polymerase I, and the resulting vector, pUC105 *Bam*<sup>-</sup> cut with *Xho*I-*Sal*I. Scanlan (1988) has shown that a *Xho*I-*Sal*I deletion did not affect transformation frequency, since the cyanobacterial replication origin of this plasmid is contained within a 4.65 kb *Bam*HI-*Xho*I fragment (Gendel, 1987). Insertion of the promoterless *lacZ* gene from pDAH274 into the large *Xho*I-*Sal*I fragment of pUC105 *Bam*<sup>-</sup> produced pLACPB2, similar to pLACPB1, but containing transcription terminators at either end of the *lacZ* gene. The multiple cloning site in front of the *lacZ* gene, contained a unique *Bam*HI site, into which cyanobacterial chromosomal DNA could be inserted.

*Synechococcus* R2 chromosomal DNA was subjected to partial *Sau*3A digestion, ran on a 0.7% agarose gel and 0.5 kb - 4 kb fragments electoeluted. These fragments were then cloned into the unique *Bam*HI site of pLACPB2, creating a 0.5 kb - 4 kb *Synechococcus* chromosomal DNA library in this vector.

A similar library was constructed in pLACPB1, and then both vectors used to transform *Synechococcus* R2-SPc and *E. coli* MC1061. *Synechococcus* R2-SPc was used for two reasons i) Scanlan (1988) reported transformation frequencies 10-100 fold higher in this strain than in *Synechococcus* R2 and ii) since it lacks the resident pUH24 plasmid, possible loss of cloned DNA through recombination events between the two plasmids are prevented,

Figure 6.4 : Construction of pLACPB2 ; A promoter probe vector for use in *Synechococcus* PCC7942



**T** : Transcription terminator

**MCS** : Multiple cloning site

estimated by Kuhlemeier et al. (1981) to occur in up to 90% of the transformed population.

Transformation frequencies (see Table 6.3) were obviously higher in *E.coli*, since by using SOB medium (see Section 2.6) cells can be made more competent. In *Synechococcus* R2-SPc, where this method is not applicable, transformation frequencies were very good using pLACPB1, with up to  $10^4$  transformants per  $\mu\text{g}$  DNA. Using pLACPB2 transformation frequencies were 10-fold lower than in pLACPB1. This may reflect the size differences of the two plasmids, pLACPB2 being 1.5 kb larger, and if the case, is borne out by the transformation frequencies of the the plasmids containing cyanobacterial chromosomal DNA 0.5 kb - 4 kb inserts, which are approximately 10 fold lower than the corresponding plasmid without any insert. Chauvat et al. (1983) found *Synechococcus* R2, as opposed to R2-SPc gave higher transformation frequencies, however the frequencies observed with R2-SPc were high enough to screen and select a variety of transformants. pUC105 and pUC303 were used in *Synechococcus* R2-SPc to assess transformation efficiency, pBR322 was used for the same purpose in *E.coli*.

The expression of *lacZ* in *Synechococcus* R2-SPc transformed with pLACPB1 and pLACPB2 containing chromosomal DNA libraries (pLACPB1::LIB and pLACPB2::LIB), under high and low  $\text{CO}_2$  conditions was investigated. The aims were twofold: i) To identify presumptive  $\text{CO}_2$ -regulated promoters and ii) To assess whether the presence of transcription terminators had any effect



Table 6.3 : Transformation frequencies of shuttle and promoter vectors in *Synechococcus* R2-SPc and *E. coli*.

Plasmid	Selection	Transformants/ $\mu$ g DNA	
		<i>E.coli</i>	<i>Synechococcus</i> R2-SPc
pUC303	Cm <sup>r</sup>	-	10 <sup>5</sup> -10 <sup>6</sup>
pUC105	Cm <sup>r</sup>	-	10 <sup>5</sup>
pBR322	Ap <sup>r</sup>	4 x 10 <sup>7</sup>	-
pLACPB1	Cm <sup>r</sup>	10 <sup>6</sup> -10 <sup>7</sup>	10 <sup>6</sup>
pLACPB2	Cm <sup>r</sup>	10 <sup>5</sup> -10 <sup>6</sup>	10 <sup>4</sup>
pLACPB1::LIB	Cm <sup>r</sup>	10 <sup>6</sup> -10 <sup>7</sup>	10 <sup>5</sup> -10 <sup>6</sup>
pLACPB2::LIB	Cm <sup>r</sup>	10 <sup>4</sup> -10 <sup>5</sup>	10 <sup>2</sup> -10 <sup>3</sup>
pDAH274	Km <sup>r</sup>	10 <sup>5</sup> -10 <sup>6</sup>	0

on endogenous *lacZ* activity.

To achieve this, *Synechococcus* R2-SPc transformed with pLACPB1::LIB and pLACPB2::LIB was replica plated onto BG-11 plates under high and low CO<sub>2</sub> conditions. Screening corresponding high and low CO<sub>2</sub> plates by spraying with MUG allowed the identification of transformant colonies which showed differential *lacZ* activity under these conditions (see Fig. 6.5). In order to ensure low CO<sub>2</sub> conditions, BG-11 plates had to be placed inside sealed gas bags, supplied with air, to ensure a positive pressure in the gas bag, otherwise corresponding high and low CO<sub>2</sub> plates showed very few differences in B-galactosidase activity.

2500 pLACPB2::LIB and 500 pLACPB1::LIB transformants were screened, and from these "interesting" transformants were selected for analysis by a quantitative fluorimetric assay, which allowed the amount of *lacZ* (and hence promoter activity) to be assessed throughout the growth cycle (see Fig. 6.6). Generally B-galactosidase activity increased throughout the growth cycle, decreasing upon reaching stationary phase, and hence in Table 6.4, activities are expressed from mid-late log phase cells, standardised for culture density.

The background expression of an intact *lacZ* gene on a multicopy plasmid in a homologous host, even lacking a recognisable promoter can apparently be quite high (Casadaban et al., 1980), and thus difficulty may be encountered in distinguishing strains carrying the desired fusion from the parent strain. It is

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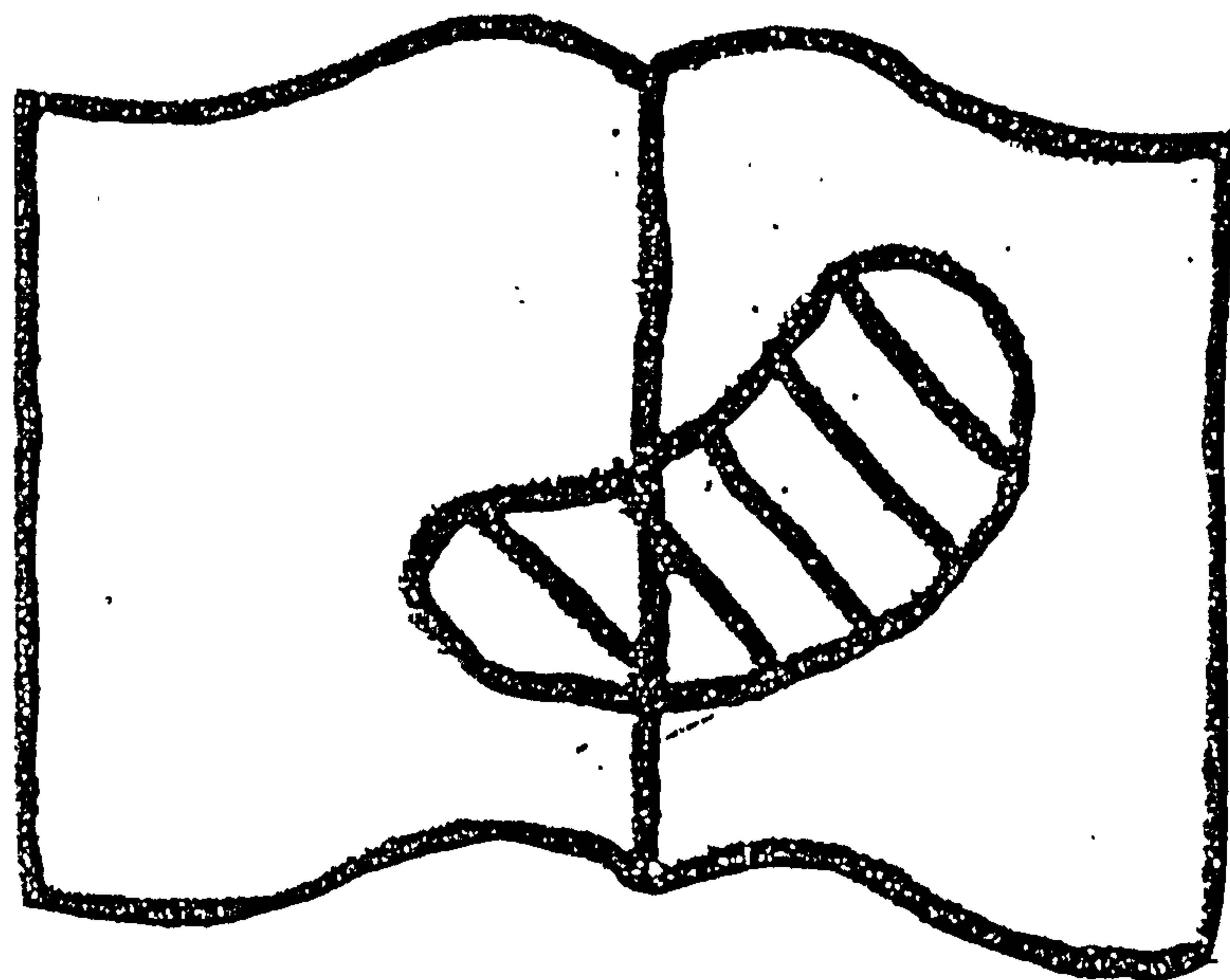




Figure 6.5 : Differential LacZ activity in *Synechococcus*  
R2-SPc : [pLACPB2] transformants

HIGH CO<sub>2</sub>

LOW CO<sub>2</sub>

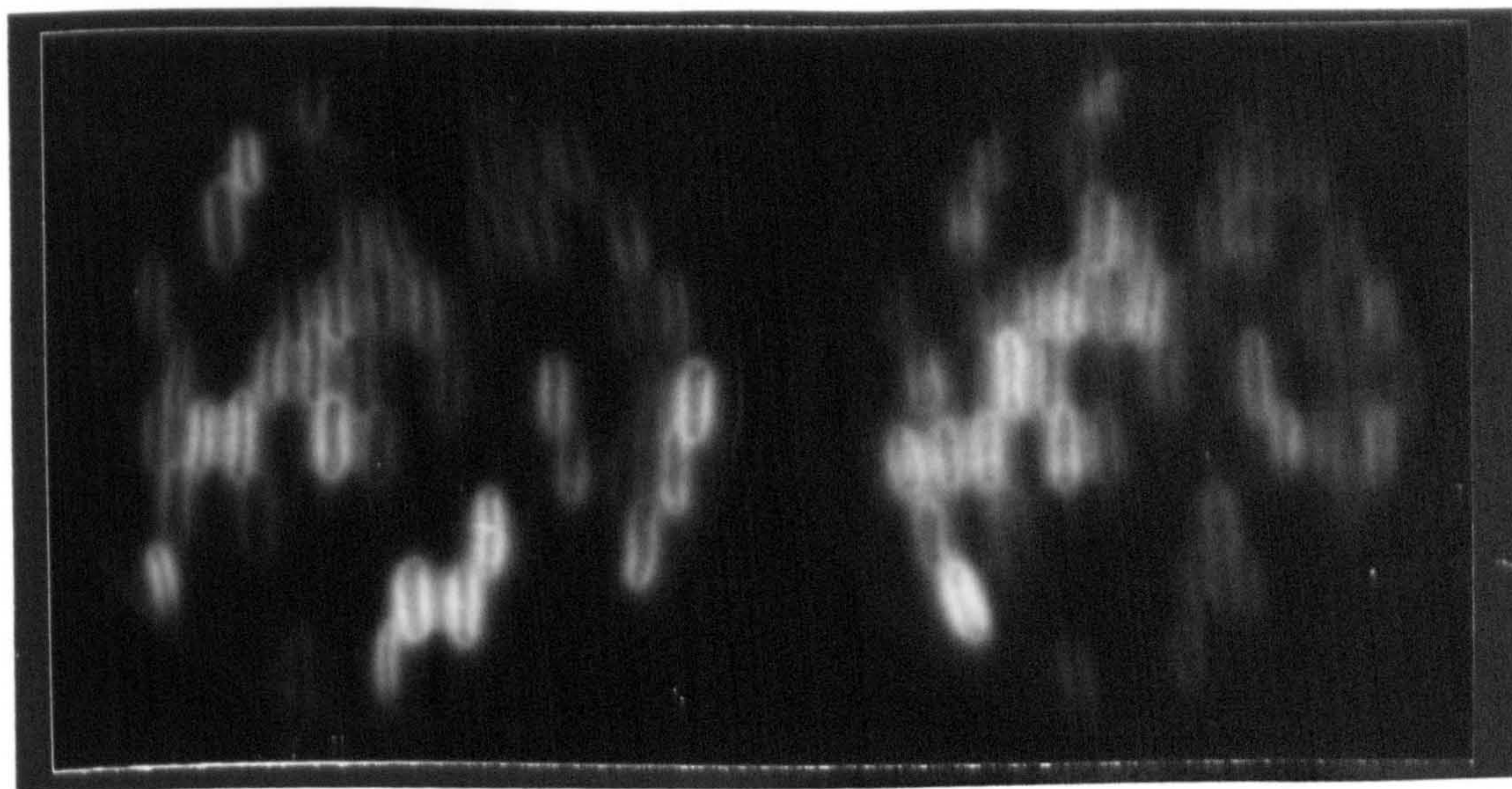
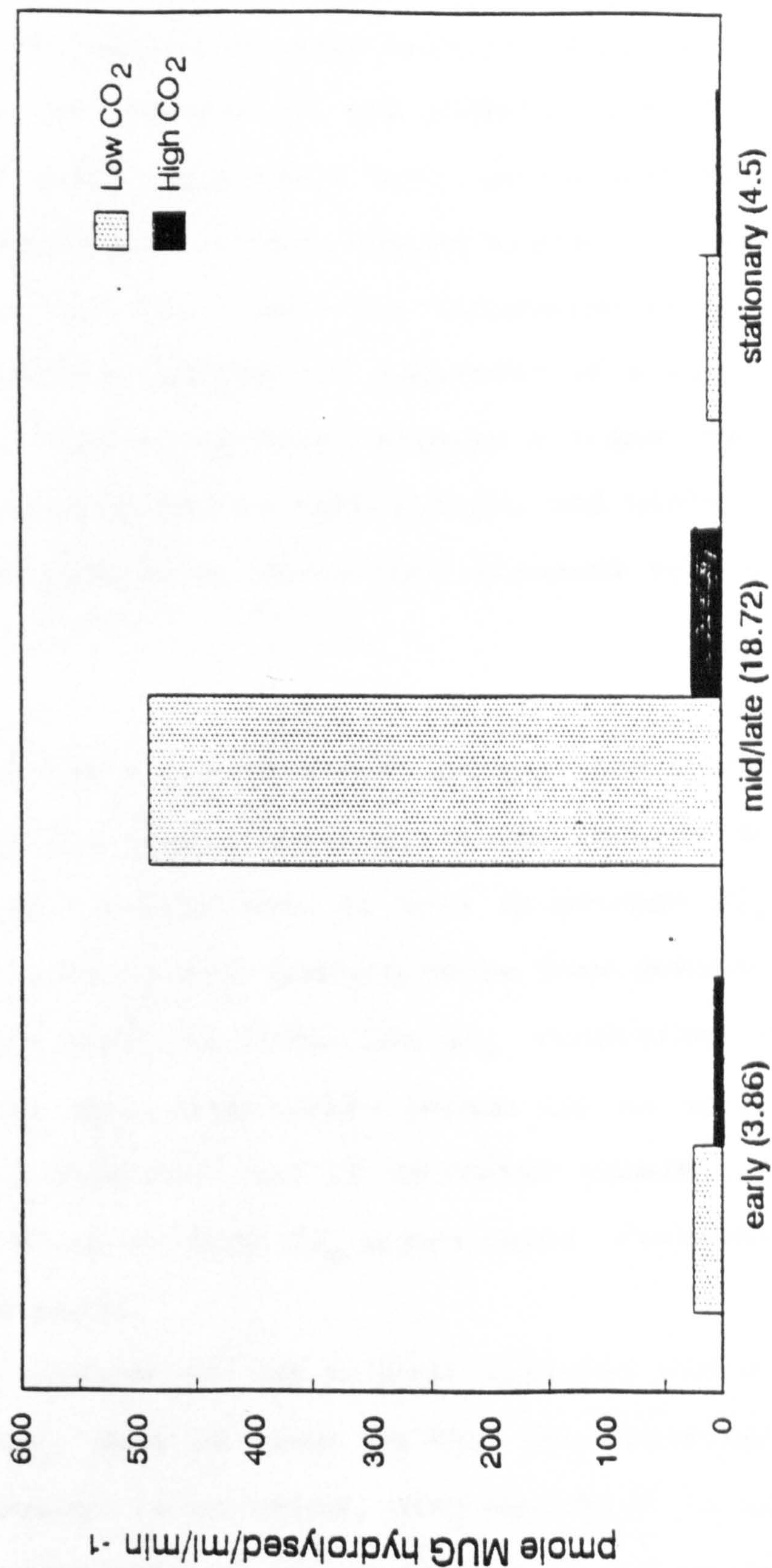




Figure 6.6 : Expression of LacZ in clone 4 (pLACPB1) during the growth cycle



Numbers in brackets are the fold differences between high and low CO<sub>2</sub> grown cultures

apparently not certain that plasmids are multicopy in cyanobacteria, and may only be present in similar numbers to the chromosome (personal communication, F.Joset). Scanlan (1988), found endogenous B-galactosidase activity associated with pLACPB1, which was repeated in the pLACPB1 control culture used in these experiments (see Table 6.4). Using pLACPB2, which contained a transcription termination signal upstream from the site of chromosomal DNA insertion, expression of B-galactosidase was reduced twofold, making the detection of a particular gene fusion easier. This difference reflects a transcriptional effect since the plasmid origin of replication, and hence copy number, is the same in each case, since both plasmids were derived from pUC105.

The results in Table 6.4 show that amongst the transformants selected, B-galactosidase activity varies considerably from transformant to transformant, as well as between CO<sub>2</sub> regimes. The fact that most of the transformants show greater B-galactosidase activity under low CO<sub>2</sub> conditions reflects the fact that these were selectively chosen for on solid media. In transformant 5 (pLACPB1) and 13 (pLACPB2) however, highest activity was found in high CO<sub>2</sub> grown cells, reflecting what was seen on solid media.

Regardless of whether or not highest activity was seen under high or low CO<sub>2</sub>, what is clear is that environmental regulation of gene expression is occurring, with variation in the CO<sub>2</sub> regime the various transformants are grown under, the stimuli.



TABLE 6.4 : Expression of LacZ in selected *Synechococcus* R2-SPc transformants grown under low and high CO<sub>2</sub> conditions

Transformant activity	βGal activity (MUG units <sup>a</sup> )		Ratio of βGal (low CO <sub>2</sub> /high CO <sub>2</sub> )
	Air level CO <sub>2</sub>	5% (v/v) CO <sub>2</sub> in air	
PLACPB1 CONTROL			
2	53	39	1.35
4	760	50	15.20
5	490	56	8.75
6	10	80	8.00
8	140	30	4.66
	500	116	4.30
PLACPB2 CONTROL			
C2	24	17	1.4
2	5	4	1.3
3	155	26	6.0
4	124	67	2.0
5	925	232	4.0
6	1955	223	9.0
9	782	89	9.0
13	148	23	6.5
15	89	157	2.0
16	79	47	2.0
17	168	26	6.0
22	423	42	10.0
	180	73	2.5
<i>Synechococcus</i> R2	0.6	0.6	1.0

<sup>a</sup>1 unit of specific activity is defined as one puomole of MUG hydrolysed per ml of culture per minute, normalised for culture density (OD<sub>750</sub>)

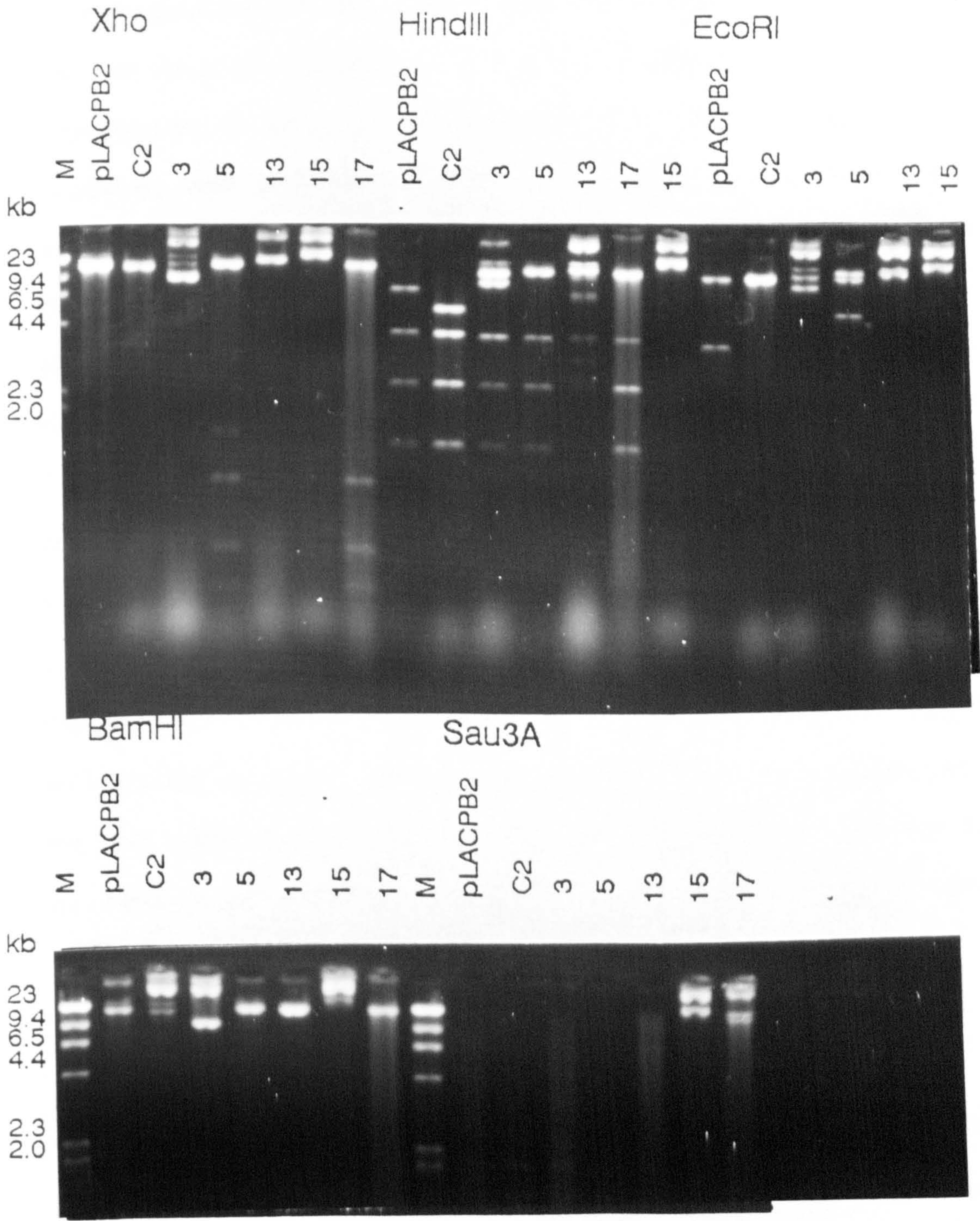
Transformant C2 was one of a few colonies screened which showed no B-galactosidase activity on solid media, looking black after MUG treatment and visualisation under u.v.light. In liquid media, it again showed very low B-galactosidase activity, lower than the control, which did not contain a *Synechococcus* R2 chromosomal DNA insert (Table 6.4). The transformant can grow on the selective media used, and the plasmid can be reisolated from the cells. Restriction endonuclease analysis of the plasmids (see Fig. 6.7) show that C2 and the parent plasmid pLACPB2 are of a similar size, it would thus appear that the *Synechococcus* R2 chromosomal DNA insert is not present. Southern blot analysis (Fig. 6.8) confirmed this, but it still does not explain why the level of B-galactosidase activity is much lower than the pLACPB2 control, and this is probably due to a spontaneous deletion in the *lacZ* gene, which could explain why *Hind* III digestion shows the transformant C2 to be slightly smaller than pLACPB2 (see Fig. 6.7).

#### 6.3.3 Expression of pLACPB2 in *Escherichia coli* MC1061

The expression in *E.coli* of the pLACPB2::*Synechococcus* R2 chromosomal DNA gene fusions shown in Table 6.4 was determined. The pLACPB2::LIB gene fusions were isolated from these transformants (Section 2.18) and used to transform *E.coli* MC1061, which has no endogenous B-galactosidase activity. Chloramphenicol (0.3 mg/ml) was added to the nutrient broth, to select for transformants. ONPG and MUG assays were performed on the transformants (see Table 6.5). It can be seen that



Figure 6.7 : Restriction endonuclease digestion of pLACPB2::  
*Synechococcus* PCC7942 chromosomal DNA  
transformants



M :  $\lambda$  HindIII markers



Synechococcus R2 chromosomal DNA will act as a promoter in *E.coli*, enabling B-galactosidase to be expressed. In fact, higher levels of *lacZ* expression were seen in *E.coli* than in *Synechococcus* R2-SPc. This can be correlated to the strength of the various promoters in *E.coli*, although the level of expression in *E.coli* is proportional to the copy number of the plasmid, and pACYC184, the *E.coli* replicon in pLACPB2 (from pUC105) is known to be multicopy in *E.coli*. As in *Synechococcus* R2-SPc *lacZ* expression was not uniform, each transformant showing differential B-galactosidase activity. The strength of these promoters do not correspond with the results for *Synechococcus* R2-SPc (see Table 6.4), where transformant 4 had the highest *lacZ* activity, since in *E.coli* transformant 3 shows the highest *lacZ* activity. Since each plasmid has the same *E.coli* origin of replication, and would therefore be expected to be present in the same copy number, it suggests that the factors affecting promoter strength within the cyanobacterial chromosome do not operate in *E.coli*, and this might in part be due to the differential recognition of the different promoter sequences by the host RNA polymerase.

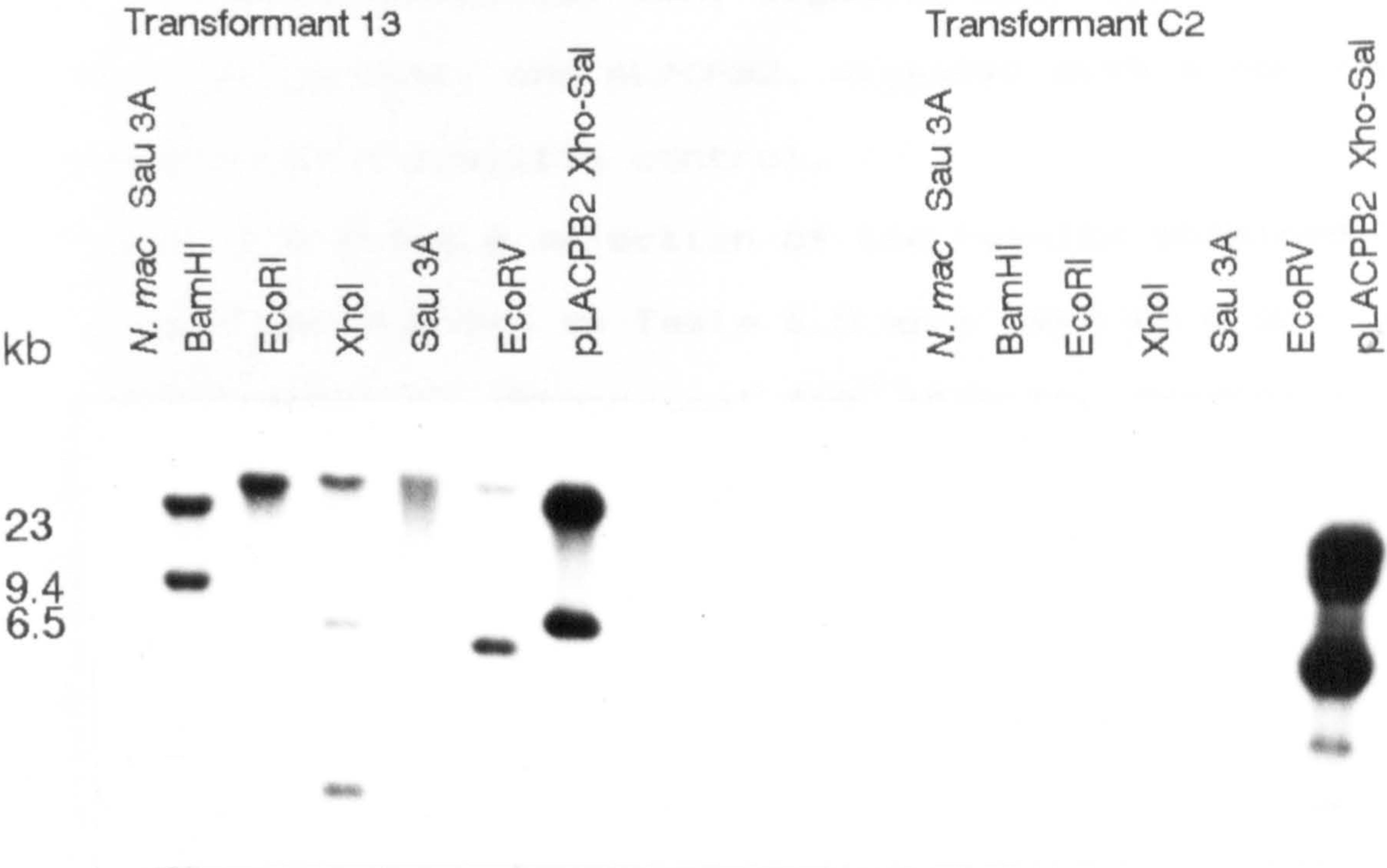
#### 6.3.4 Southern blot analysis of pLACPB2

Restriction endonuclease analysis of isolated transformant plasmids (Fig 6.7) suggested that transformant C2 was lacking any chromosomal DNA insert. It was also unclear as to whether transformant 15 contained a chromosomal DNA insert, because it proved very resistant to endonuclease digestion.

Table 6.5 : Expression of LacZ in E.coli MC1061 from promoter probe pLACPB2

Transformant	$\beta$ -Gal activity	
	MUG	ONPG $\Delta 420 \text{ min}^{-1} \text{mg protein}^{-1}$
pLACPB2	72.7	0.092
C2	13.8	0.014
2	13.8	0.19
3	2053	33.8
4	243	0.5
5	860	5.7
9	443	1.7
13	237	0.6
15	16.8	0.14
16	1834	6.3
17	1012	2.9
22	769.5	1.6

Figure 6.8 : Southern blot of *Synechococcus* PCC7942  
chromosomal DNA probed with pLACPB2::LIB





To confirm that the transformants actually contained a chromosomal DNA insert, their plasmids were digested and radiolabelled (see Section 6.2.5) and then used to probe *Synechococcus* R2 chromosomal DNA.

*Hostoc mac* chromosomal DNA, digested with *Sau*3A, was used as a negative control, and pLACPB2, digested with a variety of enzymes, as a positive control.

Figure 6.8 shows a selection of the results obtained. All of the transformants shown in Table 6.5 were used to probe *Synechococcus* R2 chromosomal DNA. With the exception of C2, all of the transformants were shown to contain chromosomal DNA inserts, and hence represent true gene fusions.

#### 6.4 Conclusion

The construction of the promoter probe vector pLACPB2, and its use along with pLACPB1 in *Synechococcus* R2-SPc in identifying a number of promoters showing differential *lacZ* expression under different CO<sub>2</sub> regimes has been described in this chapter.

The vector pLACPB2, which contains transcription terminators and showed lower endogenous *lacZ* activity than pLACPB1, was also used to transform *E.coli*. It was shown that *Synechococcus* R2 chromosomal DNA, could function as a promoter in *E.coli*, but that the strength of the promoters was not uniform between the organisms, reflecting the different pattern of DNA sequences required to control promoter strength in the two organisms.

Since a number of CO<sub>2</sub>-regulated promoters have been identified, this system could be used as an alternative to the mutant

isolation work (see Section 6.1.2) for identifying genes involved in inorganic carbon concentration. It would be a relatively simple procedure to isolate the promoter fragment, insert it into one of the pBR plasmids and then inactivate it by *in vivo* insertional mutagenesis using Tn5 (or *in vitro* using the omega fragment if the promoter is known to contain a restriction site). Any transformant growing on kanamycin after the plasmid was reintroduced into *Synechococcus* R2 would have the plasmid integrated into the host chromosome, inactivating the gene, which could be selected for under the required conditions. This technology could be used to examine any type of environmental regulation of gene expression in *Synechococcus* R2 and could be applied to other organisms, including *Synechocystis* PCC6803, which is transformable and has the advantage of being capable of photoheterotrophic growth. Shuttle and integrational vectors do exist in this organism, and one shuttle vector constructed by Ferino and Chauvat (1989) has the advantage of conferring no detectable chloramphenicol acetyl transferase activity in the absence of a promoter insert.

Concluding remarks

The effect of growth under different carbon regimes has been studied at the physiological and molecular level in unicellular cyanobacteria.

In all of the species studied capable of growth in freshwater, organisms grown at air levels of  $\text{CO}_2$  possessed an active Ci uptake and concentrating mechanism, which was inhibited to varying degrees by growth at high  $\text{CO}_2$  levels.

Induction of the Ci uptake mechanism was not an "all or nothing" response, it was found in both chemostat and batch cultures of the same organism that cells with varying affinities for Ci were seen. From the defined DIC conditions produced in the chemostat, and current research (see Badger and Gallacher, 1987) this would seem to implicate that cyanobacteria can "fine-tune" the Ci uptake mechanism in response to external Ci supply. These changes in affinity have been reported to occur over DIC concentrations often found in the natural environment, and may be of ecological significance (Mayo et al., 1986).

In three oceanic species of cyanobacteria studied, the Ci uptake mechanism was absent, under the conditions employed, following growth on both high and low  $\text{CO}_2$ . Given that these organisms inhabit an environment with a constant DIC concentration of around 2 mM, with bicarbonate responsible for 90 % of this total (Round, 1981) the selective pressure to develop and maintain the energetically expensive Ci uptake and concentrating system would be absent.

A number of presumptive  $\text{CO}_2$ -regulated promoters induced under



either high or low  $\text{CO}_2$  conditions have also been identified in this study, adding further weight to the suggestion that cyanobacteria are capable of altering gene expression and enzyme activities in response to changes in  $\text{Ci}$  supply.

The decay in  $\text{Ci}$  uptake activity was also studied. It was found in *Synechocystis* PCC6803 that addition of both  $\text{NaHCO}_3$  and glucose to low  $\text{CO}_2$ -grown cells caused a rapid decay in  $\text{Ci}$  uptake activity. Cells transferred to high  $\text{CO}_2$  conditions did not completely lose the ability to transport  $\text{Ci}$ , consistent with the view that these organisms possess an active  $\text{CO}_2$  transporting mechanism (see review by Miller et al., 1990). Gene control alone, followed by a dilution/turnover of the components of the  $\text{Ci}$  uptake mechanism is not thought to be the mechanism responsible for this decay, because of the very fast initial decay rates ( $t_{0.5}$  1.2 h) seen. It is thought that the initial event in this process probably involves cessation of gene transcription, and it has been shown in the slow growing oceanic *Synechococcus* sp. that changes in the nitrogen status of the cell caused a complete cessation of the transcript of the phycoerythrin gene within 2 hr (J. Newman per. comm.).

Transfer of low  $\text{CO}_2$ -grown *Synechocystis* PCC6803 to high  $\text{CO}_2$  or photoheterotrophic conditions resulted in the appearance of several similar phosphopolypeptides in  $^{32}\text{P}$ -orthophosphate labelled cultures, which occurred over a similar time course to the decay in  $\text{Ci}$  uptake activity, making it tempting to speculate that there is some causal involvement between the two processes. Interestingly, the glucose analogue, DOG was found to cause a

similar decay in the  $C_i$  uptake mechanism to that seen following addition of glucose, but it did not affect the phosphorylation pattern seen. It has been hypothesized in this study that a signal for the switch-off of the  $C_i$  uptake mechanism involves accumulation of a pool of glucose-6-phosphate. Addition of the glucose analogue DMG to low  $CO_2$ -grown *Synechocystis* PCC6803 did not cause a decay in the  $C_i$  uptake mechanism and it is believed the structure of this analogue, with its methyl substitution, prevents entry into the active site of hexokinase. This hypothesis has been given further credence by the observation that glucose analogues blocked at the 6' position do not cause a decay in the  $C_i$  uptake mechanism in *Synechocystis* PCC6803 (N. Silman per. comm.).

The actual role played by phosphorylation in this regulatory process is not as yet known, however work in this laboratory has shown that transfer to the dark caused decay in  $C_i$  uptake activity and produced a similar pattern of phosphopolypeptides (N. Silman per. comm.). Phosphorylation has been shown to modulate carbon metabolism in chloroplasts of *Spinacia oleracea* (Foyer, 1985), and the phosphorylation of several thylakoid proteins in the same plant were markedly affected by the bicarbonate concentration supplied (Sundby et al., 1989). The regulatory role of phosphorylation therefore requires further clarification, and the identification of the phosphopolypeptides would certainly help in this process.

RuBisCO activity was studied in batch and chemostat cultures. In

batch cultures, alteration in the activity of RuBisCO would not seem to be a mechanism cyanobacteria employ to adjust to depletion of  $C_i$  in the external environment, and in fact where differences in activity did occur, greater activities were seen in high  $CO_2$ -grown cells. In chemostat cultures, there was a discontinuous increase in RuBisCO activity as the external DIC concentration decreased, suggesting some kind of enzyme activation/stabilization as opposed to transcriptional control. Future work in chemostats will need to look at the response of the organism to  $C_i$  depletion at external DIC concentrations which produce cells which have a fully induced  $C_i$  uptake mechanism, as in this study external DIC concentrations were used which produced cells with intermediate affinities for  $C_i$ .

The use of  $CO_2$ -regulated promoters to identify genes involved in the  $C_i$  uptake mechanism has been discussed. At present the only gene identified as playing an essential role in the  $C_i$  uptake process is one homologous to the subunit-2 gene of NADH-dehydrogenase in *Synechocystis* PCC6803 (Ogawa, 1991b).

In a wider context, the work performed elucidating the  $C_i$  uptake and concentrating process in cyanobacteria and green algae may also prove to have agricultural relevance in  $C_3$  higher plants. Although a few workers have suggested that active  $C_i$  transport may be involved in these terrestrial  $C_3$  species (Volokita et al., 1981; Machler et al., 1985; Machler et al., 1986), the bulk of evidence points to the fact that carboxylation of RuBP,



mediated by RuBisCO is the first step in the reductive assimilation of  $\text{CO}_2$ , with little or no biochemical action preceeding this event.

It is well documented that supplying  $\text{C}_3$  plants with an enhanced  $\text{CO}_2$  supply leads to an increased yield. Kimball, (1983) using an assemblage of previous observation reported that agricultural yields would probably rise by 33% with a doubling of atmospheric  $\text{CO}_2$  concentration. In wheat, it has been found that the greatest seed production occurred in wheat cultivars subjected to ambient  $\text{O}_2$ /high  $\text{CO}_2$  treatment (Musgrave and Strain, 1988).

As ancestry as well as contemporary counterparts of the eukaryotic chloroplast, there is the possibility that once the genes involved in  $\text{Ci}$  uptake have been identified, they can be transferred to economically important  $\text{C}_3$  plants with the hope that improved yields of agriculturally important crops will be produced.

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Karagouni, A.D., Bloye, S.A. & Carr, N.G. (1990). The presence and absence of inorganic carbon concentrating systems in unicellular cyanobacteria. FEMS Microbiol. Lett. 68, 137-142.

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